# CORRELATION OF RESAZURIN AND METHYLENE BLUE THIOCYANATE REDUCTION TESTS ON RAW MILK BY MODIFIED & STANDARD METHODS

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BY

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OUTLINE INDEX

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# PREFACE

The methylene blue test for the control of the sanitary quality of raw milk has been in use for over twenty years. With the recent development of the resazurin test and its adoption by some foreign countries and by several health departments in the United States, controversies have arisen concerning the relative merits of the two tests. The possibility that the resazurin test is a more sensitive and quicker method of determining the quality of raw market milk has been of increasing interest to the Wichita-Sedgwick County Health Department and the Bacteriology Department of the University of Wichita. An attempt to correlate the results of several of the commonly used tests was initiated under the dual sponsorship of the University of Wichita and the Wichita-Sedgwick County Health Department, in order that the relative merits of the tests might be ascertained.

# CORRELATION OF RESAZURIN AND METHYLENE BLUE THIOCYANATE REDUCTION TESTS ON RAW MILK BY MODIFIED AND STANDARD METHODS

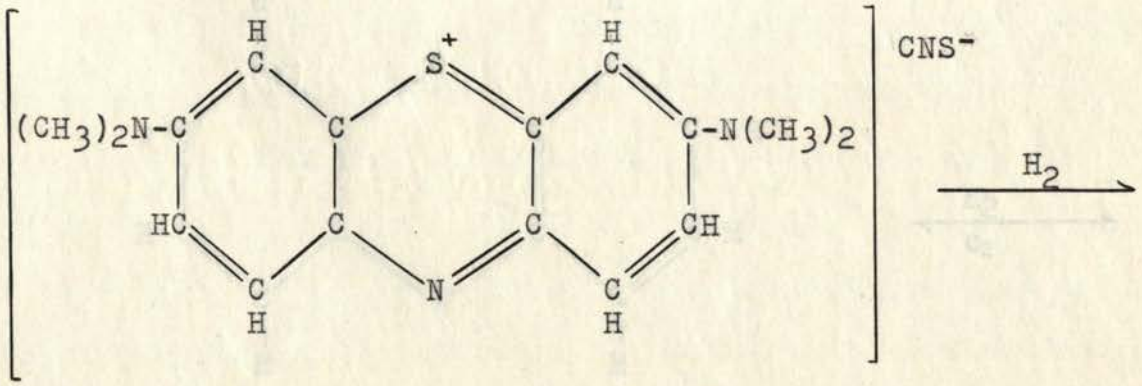
Early workers believed the phenomenon of methylene blue reduction in milk to be due to enzymatic action, hence the name, "Reductase Test". It was assumed that the reductase was elaborated by the growing bacteria. However, the enzyme theory of reduction has not been a satisfactory one and leaves unexplained such phenomena as the reduction of dyes in sterile milk and various bacteriological media. Enzymes are believed to be found only where protoplasm exists, or has existed. The constituents of the protoplasm of microorganisms necessarily are derived from the media on which they are grown. Some media have been shown to have reducing properties. The indications are that these reducing properties are imparted to the media by specific chemical constituents. It is reasonable to attribute protoplasmic reduction to chemical compounds as simple and definite as those found in bacteriological substrates. The reducing characteristics usually ascribed to enzymes are met in common media. If the reduction of dyes in milk is accomplished by enzymatic action, then the exact function of the enzymes is unknown and the conception of the heat lability of enzymes at 100 C. would have to be revised(15).

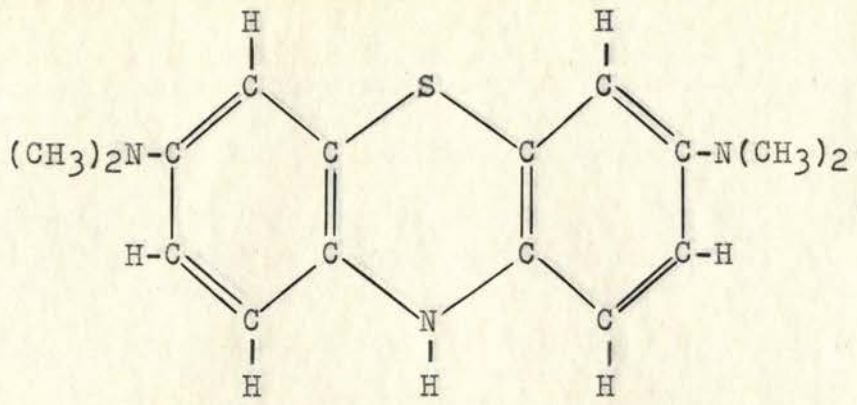
Bacterial reduction of methylene blue in milk has been reported in concentrations as high as 1:3000. In 1 ml. of such milk there would be 0.000333 gram of dye. If it be

assumed that 100 million bacterial cells are present in each milliliter of milk at the time of reduction, and that 100 million bacterial cells weigh 0.0001 gram, then a much greater weight of dye than of bacteria would be present. It is inconceivable that this amount of dye could be concentrated in the living cells. Although it is probable that all protoplasm has some reducing power, it is highly improbable that the major reduction of the dye in the methylene blue tests takes place within the bacterial cell or at the surface of the cell(15).

It is a common observation that the blue color returns to reduced methylene blue milk mixtures on shaking with air. That this is due to the oxygen in the air is proved by the fact that neither hydrogen, nitrogen nor carbon dioxide will cause the change of methylene white to methylene blue in milk, while oxygen will do so. Such a rapid return of the blue color to the milk on shaking a reduced sample with air would be impossible if the oxidative process takes place within, or at the surface of the cells(15).

The accepted reaction for the reduction of methylene blue to methylene white is given below:

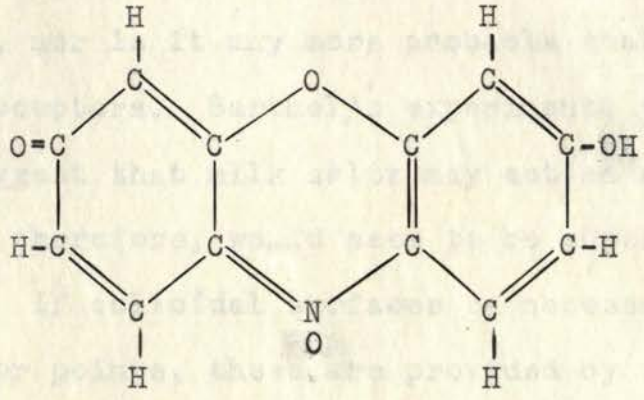




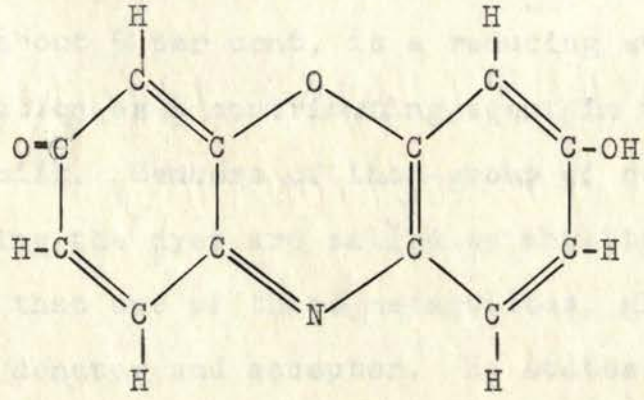

- HCNS

It will be seen from this reaction that the reduction of this dye involves no loss of oxygen, since there is no oxygen in the methylene blue molecule.

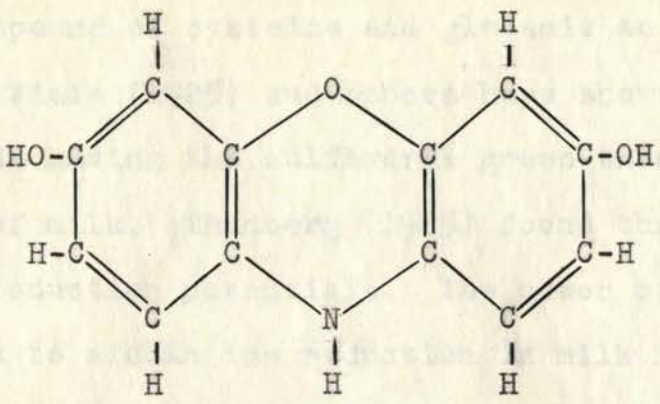
Ramsdell and Johnson(13) give the following reaction for the reduction of resazurin blue to resorufin pink and to hydrosorufin white:




$H_2$ →



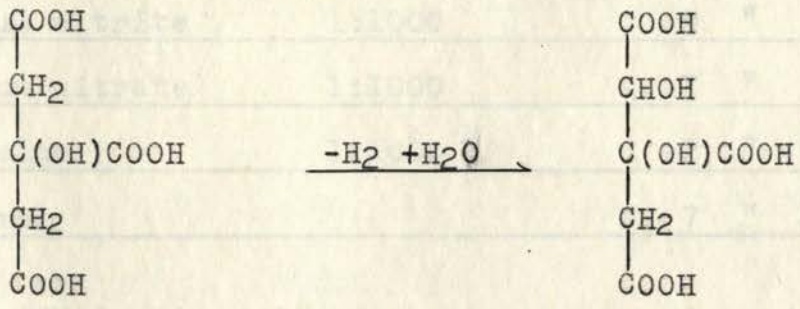

$H_2$ →
$O_2$ ←



From this reaction it will be seen that the reduction of resazurin involves a loss of oxygen from the molecule. The reaction is reversible from white to pink but not from pink to blue.

These reactions present the interesting problem of the source of the hydrogen which is involved. In the light of present knowledge it is improbable that enzymes are hydrogen donators, nor is it any more probable that they act as oxygen acceptors. Barthel's experiments with "synthetic milk" suggest that milk salts may act as catalyzers(15). Enzymes, therefore, would seem to be superfluous for this purpose. If colloidal surfaces be necessary for reduction centers or points, these are provided by the natural colloids of the milk. Lactose, which is present in milk to the extent of about 5 per cent, is a reducing sugar and must receive consideration as a contributing agent in the reduction of dyes in milk. Members of that group of compounds capable of reducing the dyes are called metabolites. Hopkins (1925) believes that one of these metabolites, glutathione, is a hydrogen donator and acceptor. He states that glutathione

is a compound of cysteine and glutamic acid with a free S-H group. Viale (1925) and others have shown that other such compounds having the sulfhydryl group enhance the reducing powers of milk. Thunberg (1925) found that succinates affect reduction potentials. The power of succinates and citrates to aid in the reduction in milk has been demonstrated by Barthel (1925). He visualizes the following reaction for citrate as a hydrogen donator in milk(15):




This reaction, he thinks, is catalyzed by the milk salts. His experiments show increasing acceleration of the reduction of methylene blue in milk on the addition of increasing amounts of sodium citrate or succinate. Quastel (1926), using the methylene blue technique, examined 103 substances as possible donators or acceptors of hydorgen in the presence of bacteria (E. coli), and fifty-six of the substances were activated(15).

The question, therefore, of the hydrogen source in the reducing processes in milk is not settled yet. It seems probable that a number of the constituents of the milk are concerned. For the practical application of the methylene blue or resazurin tests for quality in milk this is of no

great importance, since the inherent reduction "capacity" of milk is not over-taxed by the concentration of the dye used in these tests(15). Table 1 lists some substances which have an effect upon reduction.

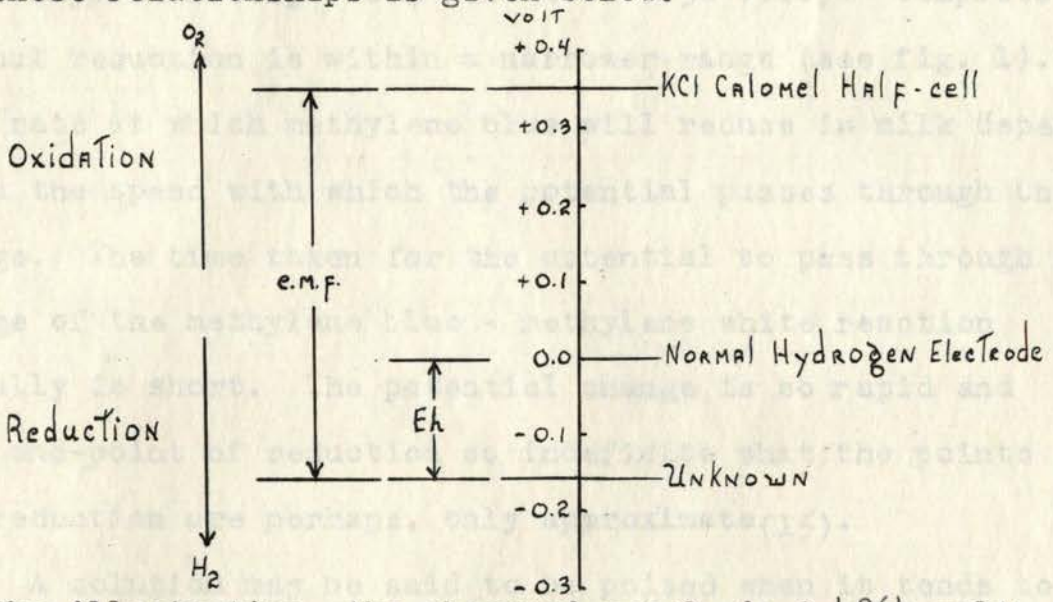
Table 1.

| | Concentration | Reduction Time |
|---|---|---|
| Sodium citrate | 1:1000 | 7 hr. 30 min. |
| Sodium citrate | 1:500 | 7 " 30 " |
| Sodium nitrite | 1:1000 | 9 " 30 " |
| Sodium nitrate | 1:1000 | 7 " 30 " |
| Cysteine | 1:1000 | 2 " 00 " |
| Control | | 7 " 45 " |

Oxidation is defined as the process in which a substance takes up positive, or parts with negative charges; while reduction is the process in which a substance takes up negative, or parts with positive charges. These electronic charges may be followed in milk potentiometrically. If an electrode of one of the regal metals is immersed in milk and a connection is made with a potassium chloride calomel half-cell through a potentiometer, and the circuit completed through a potassium agar bridge, the potential difference between the milk and the calomel half-cell is easily measured* This potential may be referred to the normal hydrogen electrode, the potential of which is arbitrarily assumed to be zero. The difference in potential between the normal hydrogen electrode and the milk is termed Eh. A schematic representation

*This apparatus has been modified by Johns and Howson(11).

of these relationships is given below:




In the illustration, the observed e.m.f. is 0.4864 volt, the unknown being negative to the calomel cell. The Eh therefore, is - 0.15 volt(15).

Methylene blue decolorizes in a zone about 0.1 volt more positive than the theoretical for methylene blue at pH 6.2 (see Clark, 1925) (15). This indicates a salt effect, or the influence of another oxidation-reduction system or systems. This effect is not constant, but varies in different milks and, at present, caution should be used in interpreting reduction intensities in organic complexes, in terms of potential, on the basis of dye reduction. Complete visual reduction (white) of methylene blue in different milks was observed at Eh values as low as + 0.075 volt and as high as + 0.225 volt(15).

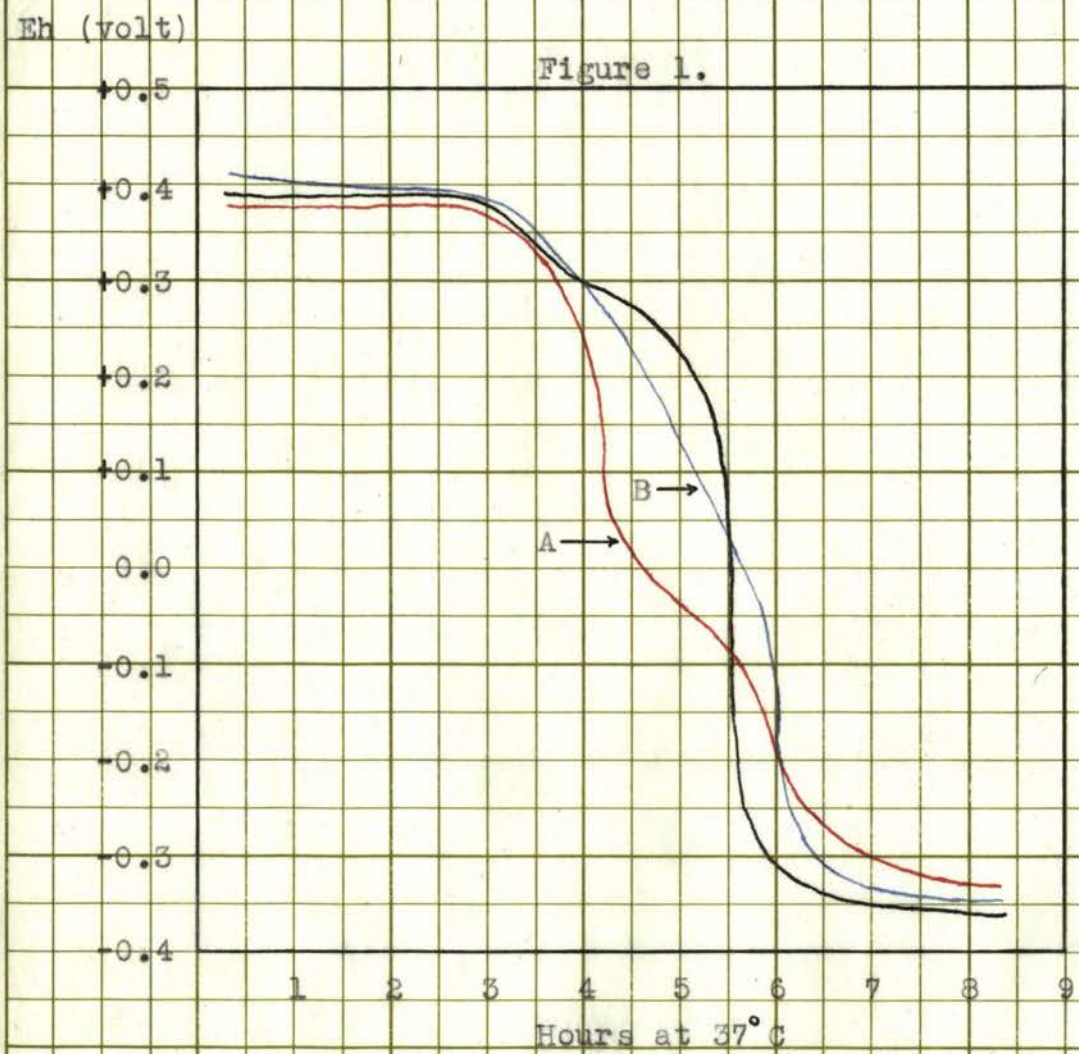
The reduction of dye in the standard concentration is rapid. The potential range of milk, within which methylene

blue changes from 4 per cent to 96 per cent reduced, is wide (approximately + 0.4 volt to - 0.36 volt). Complete visual reduction is within a narrower range (see fig. 1). The rate at which methylene blue will reduce in milk depends upon the speed with which the potential passes through this range. The time taken for the potential to pass through the range of the methylene blue - methylene white reaction usually is short. The potential change is so rapid and the end-point of reduction so indefinite that the points of reduction are perhaps, only approximate(15).

A solution may be said to be poised when it tends to resist change in Eh, on addition of an oxidizing or reducing agent. A slight poising effect of methylene blue in milk is revealed, but this effect is so small as to be almost negligible, when the standard dye concentration is used(15).

Johns and Howson(11) noted that the curve for milk plus resazurin shows a sharper initial drop in Eh than do those for milk plus methylene blue, or plain milk (fig. 1). This drop is followed by a flattening of the curve, starting at a point near that at which the full pink color appears (resazurin color changes are from blue to mauve to purple to pink to white). Later the curve again declines, reaching the same final Eh level as the others. This flattening of the curve, after reaching the pink stage, suggests that resazurin exerts a stronger poising action than does methylene blue.

Figure 1.




—— No dye in the milk.
—— Resazurin in the milk.
—— Methylene blue in the milk.

A - Point at which pink appeared.
B - Point at which white appeared.

Potentiometric studies by Johns(9), with a wide varity of milks, failed to support the view that resazurin exerts a poising effect strong enough, in market milks, to complicate the test, or to interfere with the interpretation of the results. He found no evidence to support the claim that differences in the poising properties of different milks are of sufficient magnitude to affect the results of the test.

The disappearance of methylene blue in raw milk takes place in two parallel stages (Barthel), viz.: (1) the removal of the dissolved oxygen by bacteria, and (2) the reduction of the dye by constituents of the milk(15). The reducing properties of raw milk are sufficient to account for the reduction of the dye without the aid of bacteria. The reduction "capacity" of milk is the amount of dye which the milk will reduce when the potential or "intensity" is such that reduction is possible. If the reduction capacity of milk is sufficiently large to allow the reduction of dye in a 1:100,000 concentration in the same time as a 1:200,000 concentration, then accurate measurements of the milk samples, within these limits, is not of great importance. Small errors in measurements necessarily occur in practice. There may be a slight increase in reduction time with an increase in the concentration of methylene blue, but the concentration must be approximately doubled before an appreciable increase in reduction time is observed. Any considerable increase in the

reduction time of a dye concentration of 1:100,000 over that of a concentration of 1:200,000 may be due to the antiseptic effect of the dye. The reduction "capacity" of raw milk is sufficient to take care of double the dye concentration usually employed in the test. The milk itself assists the bacteria in fixing free oxygen, but this process is too slow to be of great importance in the reduction test, as ordinarily employed(15).

Aeration before the addition of methylene blue was studied by Thornton and Hastings(16). In one experiment milk was shaken for fifteen minutes, and in another oxygen was bubbled through milk for five minutes prior to the addition of methylene blue. In neither experiment was the reduction time appreciably affected. These results show that the original milk was in approximate equilibrium with the air but, when the potential has reached the negative limit, minute accounts of oxygen will cause the potential to swing rapidly toward the positive side. This sensitivity toward oxygen appears to increase with the fall in potential. It seems reasonable to suppose that anaerobic conditions are not reached at the negative potential limit, but that an equilibrium is established at this point, and the oxygen content of the milk remains constant. It is apparent, therefore, that the reduction of methylene blue by a culture is not necessarily an indication of anaerobic conditions, but merely that a certain partial pressure of oxygen has been reached. It is probable that the effect of oxygen upon the reduction

intensities of different milks and media is not quantitatively identical(15). Thornton and Hastings(16) observed nothing which would lead them to believe that differences in the oxygen content of milks produced in the ordinary way will introduce serious inaccuracies into the test. It can be assumed, therefore, that the reduction times of methylene blue in milk, as observed in the standard application of this test, are influenced almost entirely by the rate of oxygen absorption by the milk bacteria.

There are other factors to be considered when either resazurin or methylene blue is used. The rising of cream to the top of the milk introduces an undesirable factor, since it increases the time in which the end-point is reached, by removing a number of the bacteria. When this condition exists, reduction of the dye in the rest of the milk takes longer than it would were the cream evenly dispersed throughout the milk. This would lead to the assignment of a higher grade to the milk sample than normally would be given. Therefore, any practical modification of technique which will maintain a more uniform dispersion of organisms will yield results more nearly approaching the true reduction time(10). Inversion (up-ending the tube to remix the cream and milk) restores the normal dispersion of cream, and the reduction of the dye is constant throughout the milk. The influence of inversion upon the time-potential curve causes little or no change during the first period, when the curve is approximately horizontal. As the Eh drops, however, the effect of

inversion becomes more pronounced, although the Eh changes are of a transient nature, and have little influence upon the general trend of the curve. On the other hand, dispersion of the cream layer, and associated bacteria, frequently results in much earlier reduction of both resazurin and methylene blue. That this may be due to more active growth is indicated by the higher hydrogen ion concentration in mixed tubes, at the end of the run(11). Davis(2) found that, using standard procedures, the accompanying aeration has little retarding effect on the reduction.

Chemicals such as formaldehyde and chlorine, when added to milk, may cause a lowered reduction time, if present in sufficient quantities to be bacteriostatic.

Temperature has variable effects upon resazurin and methylene blue, dependent upon the optimum temperature of the bacteria present. Thornton and Hastings(16) observed that inaccuracies in reduction tests, due to small fluctuations about the temperature of 37° C., would be comparatively small. Although 37° C. is the usual temperature employed, lower temperatures have been advocated by Davis and Watson(4). They believe that a lower temperature may more nearly approximate the conditions under which milk generally is kept. Also, bacteria which grow at 37° C. may not grow as well at a lower temperature, and vice versa. Actually, lower temperatures tend to increase the reduction time, so that resazurin alone is very adaptable to low temperatures.

Whitehead(17) observed that sunlight may catalyze an

oxidation-reduction reaction in which unsaturated fats are oxidized, and methylene blue and resazurin reduced. A reversal of color from white to blue is characteristic of exposure of methylene blue to light, and resazurin is rapidly reduced to pink. If a large number of tests are to be read, then it is advisable to read only a few at a time, keeping the rest protected from light. Otherwise, the tests suffering protracted exposure will have their reduction times changed abnormally.

The change of pH during the test is slight. It has been observed that any variations in the pH of milks to which this test is applicable are not sufficient to have any measurable visual effect upon reduction time. Hastings and Evans reported that the number of bacteria must reach approximately 100 million before any appreciable change is noted in the reaction of the milk, as measured by titration; although the bacterial content at the moment of reduction may be high, it is not great enough to cause any appreciable change in the reaction of the milk(15). The final pH value of the methylene blue is lower than that of resazurin indicating that resazurin exerts a slightly greater bacteriostatic influence than does methylene blue(11).

A knowledge of the relation of species and numbers of milk organisms to the oxidation-reduction potential would be of value in the interpretation of results with milks in which these organisms predominate. This knowledge must necessarily precede the study of milk which contains more than one species

of bacteria. The changes in the oxidation-reduction potential of milk by various milk organisms in pure culture were determined and plotted by Frazier and Whittier(6), and direct microscopic counts of the numbers of organisms present were correlated with the changes in oxidation-reduction potential, and the growth of the bacteria during that time, are shown in figures 2 to 7.

In figure 2, are shown results with one strain of Escherichia coli, at 25° and at 37° C. The curves of E. coli at 37° C. bring out the fact that the Eh curve starts to flatten at about the time that the growth curve begins to rise rapidly. Also, figure 2 shows that both rate of growth and potential vary when E. coli is grown at different temperatures. When aerated, a greater number of bacteria must be present before the bottom of the Eh curve is reached.

Figure 3 shows the results obtained with two strains of Streptococcus lactis grown at 37° C. The Eh curves of S. lactis usually show a period of fairly constant potential, followed by a sudden drop to a negative value of - 0.20 volt. The rapid rise in numbers of bacteria is almost coincident with the final flattening of the Eh curve, at which point the Eh values begin to drop very slowly. Note the extreme differences in potential and growth of two strains growing under comparable conditions.

Figure 4 shows that the Clostridium welchii culture had a long lag period and showed no increase in numbers until about the ninth hour after inoculation, this hour being plotted as the zero hour. A slow change in potential to a value only

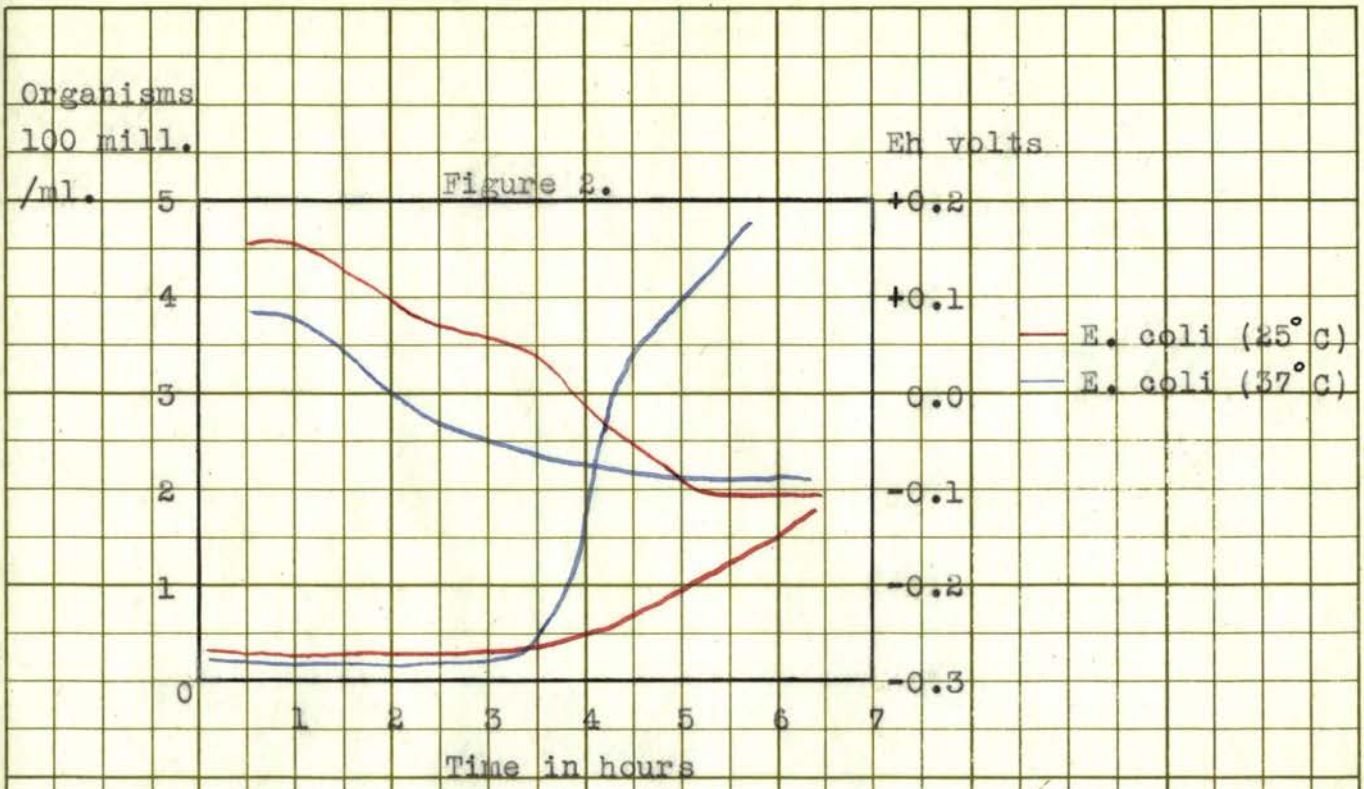



Figure 2.

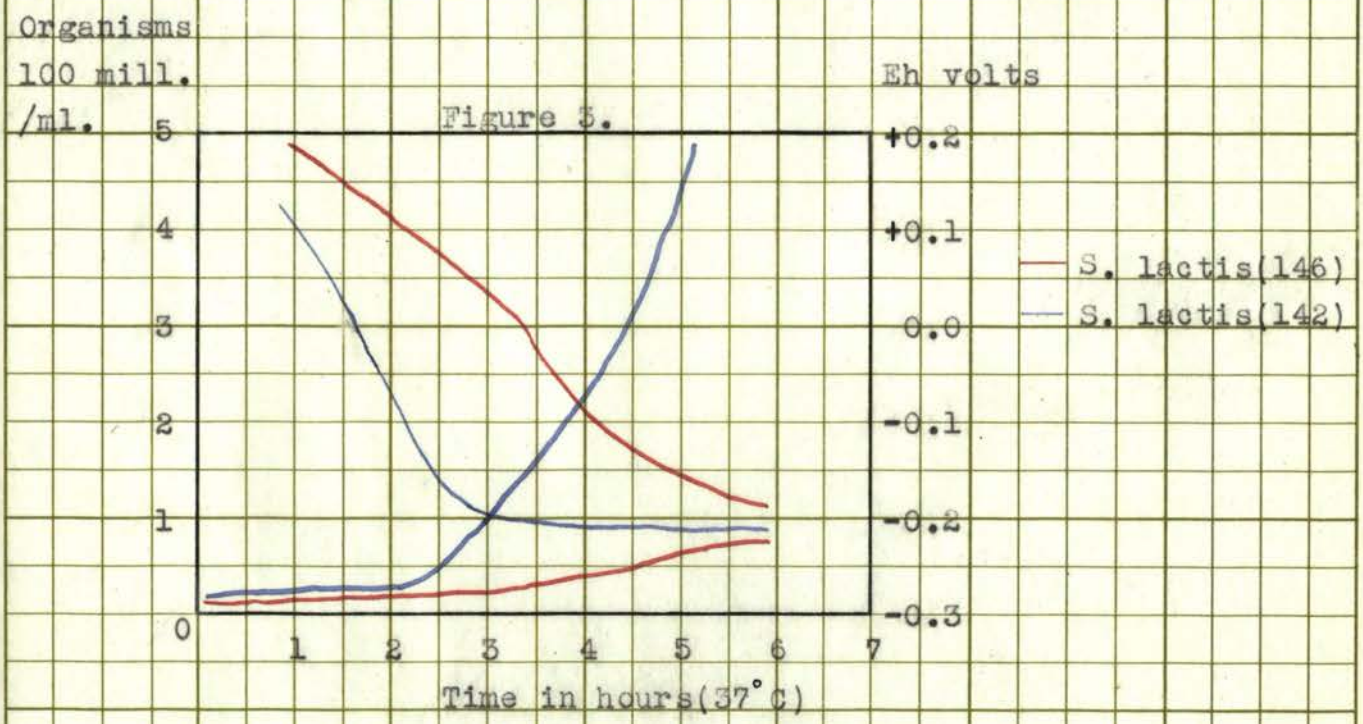



Figure 3.

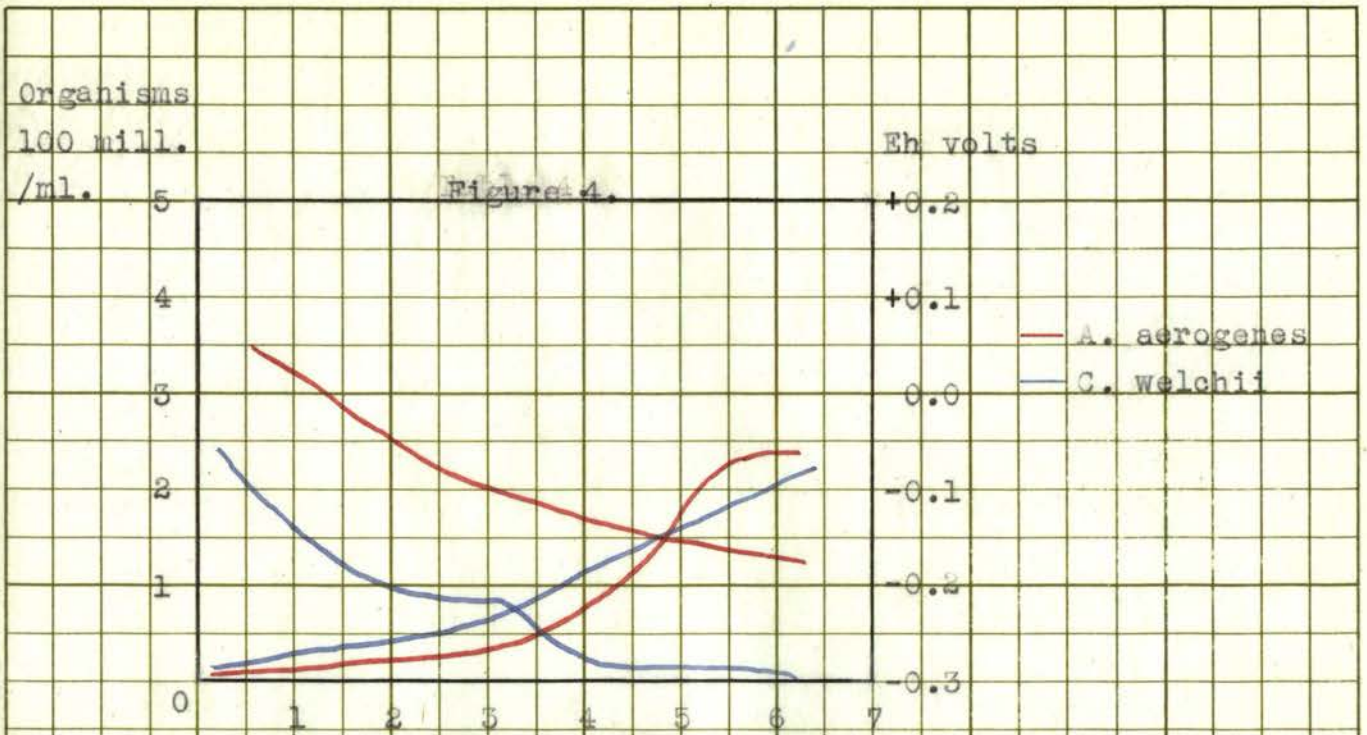



Figure 4.

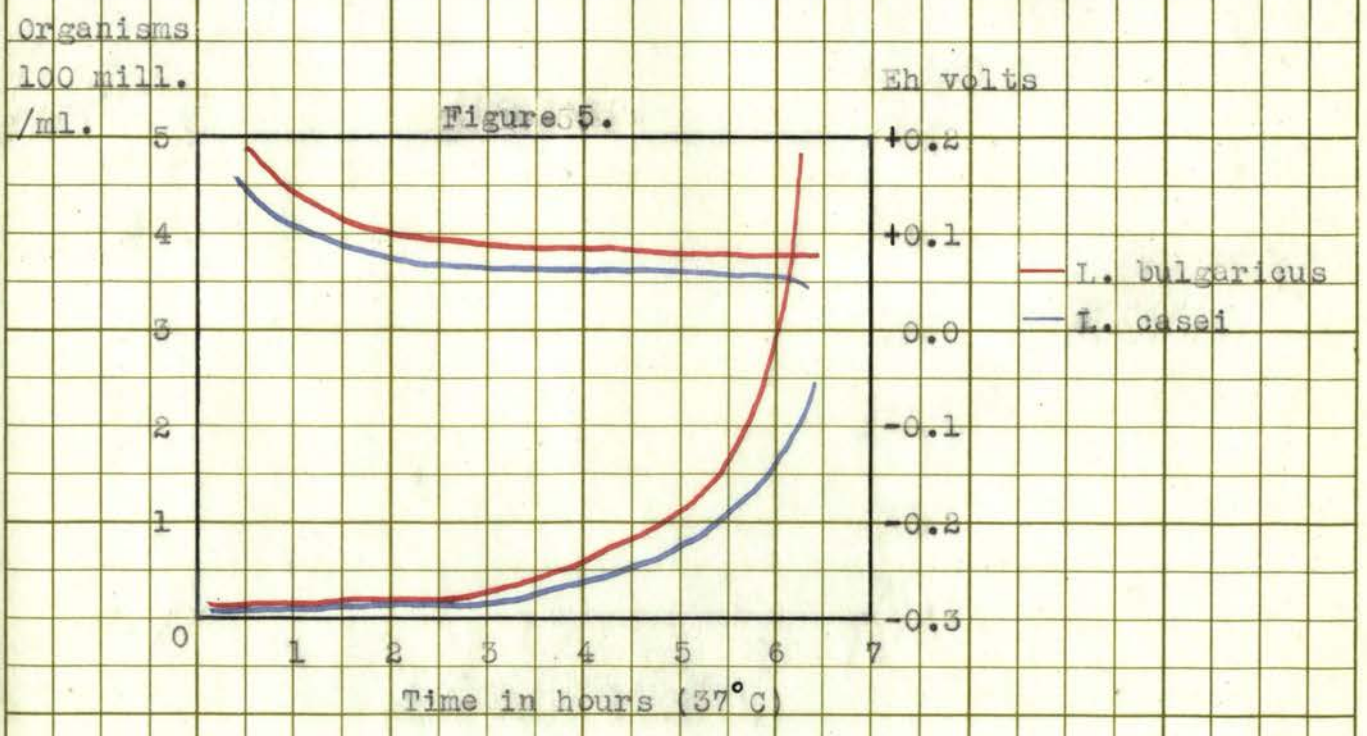



Figure 5.

slightly less negative than that given by S. lactis in milk was brought about by Aerobacter aerogenes. Bubbline air through cultures of this organism had comparatively little effect on the potential level.

Figure 5 shows that Eh curves for Lactobacillus bulgaricus and Lactobacillus casei are very s imilar. It usually takes several days, even at optimum temperatures, to attain their final Eh value of approximately - 0.235 volt in milk.

Figure 6 shows the Eh and growth curves of Streptococcus fecalis and Streptococcus thermophilus at 37° C. With both of these organisms it will be noted that considerable numbers of bacteria were present before the Eh curve had reached its lowest level. The Eh curve had only begun to drop when the growth curve started to rise. The Eh curve of S. fecalis approached practically the same limit in milk as the curve of S. lactis, but the change was more gradual.

Bacillus subtilis and Bacillus albolactis (fig. 7) grew very slowly in milk at 37° C. and the Eh values show a correspondingly slow and gradual drop. The final Eh value for both cultures after twenty four hours is very little below the value obtained at about six hours.

With many of the organisms studied, the end of the rapid drop in oxidation-reduction potential was almost coincident with the beginning of the rapid rise in numbers of bacteria. With S. fecalis and S. thermophilus, it apparently is essential that there be present comparatively large numbers of

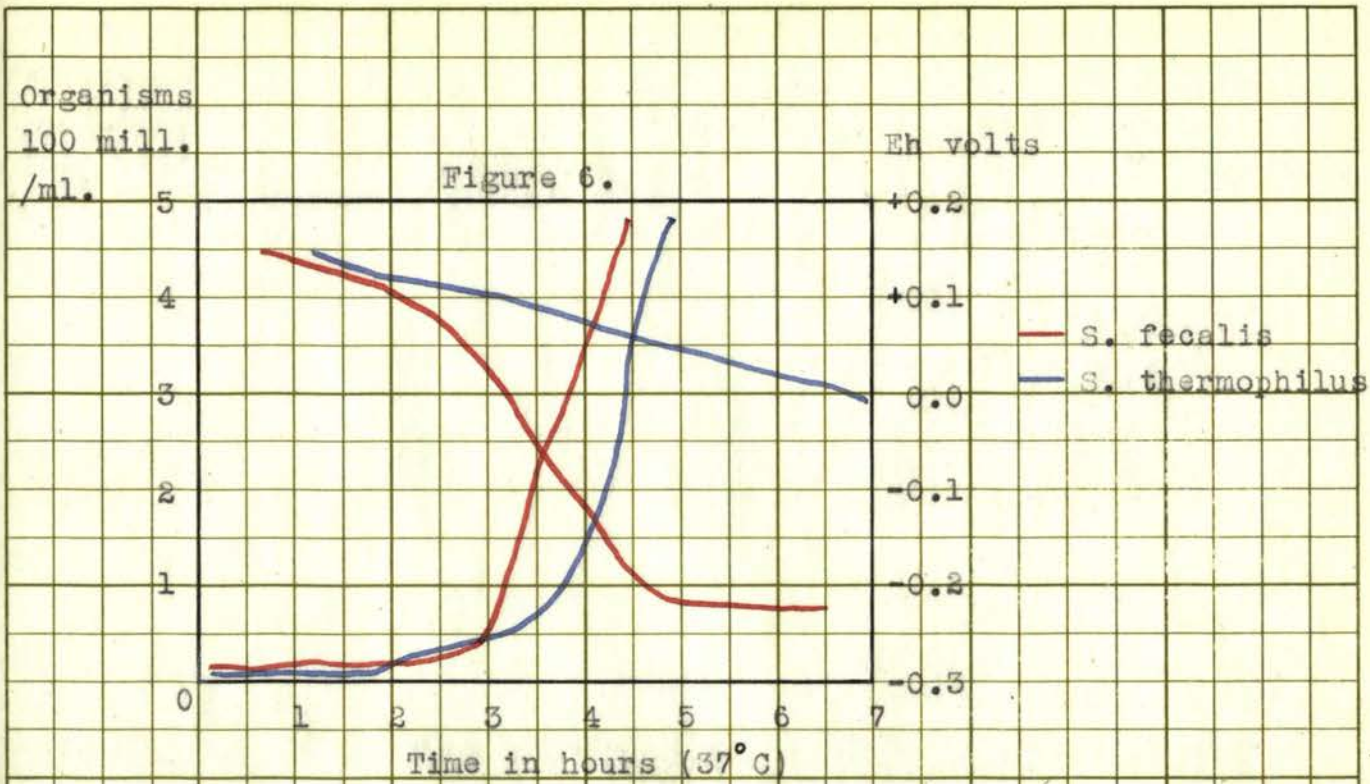



Figure 6.

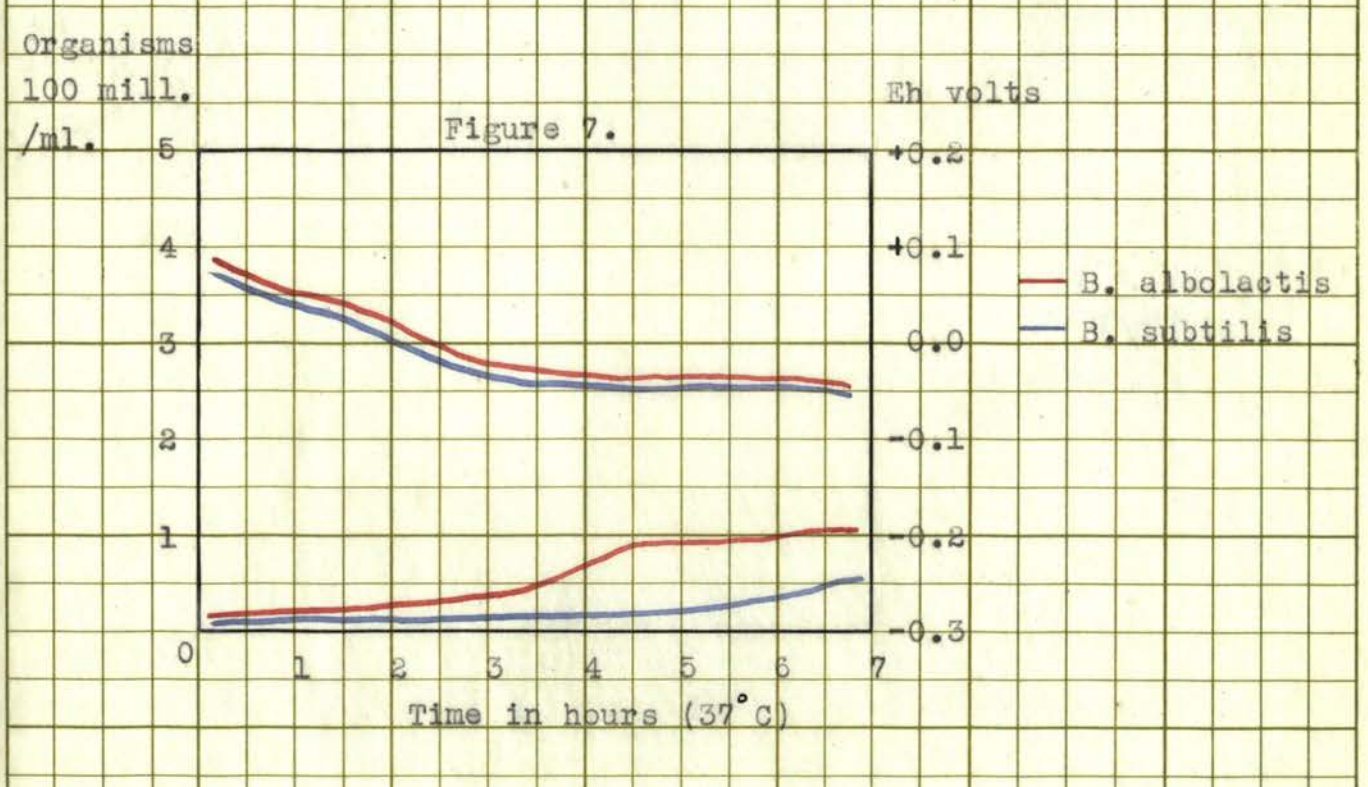



Figure 7.

actively growing organisms if the oxidation-reduction potential is to reach its greatest negative value. These organisms might be said to produce a system with a weak reducing factor.

Note that the strain of bacteria present in milk (fig. 3) determines the Eh curve that will be produced. Also, the temperature (fig. 2) employed will have considerable effect upon the Eh curve.

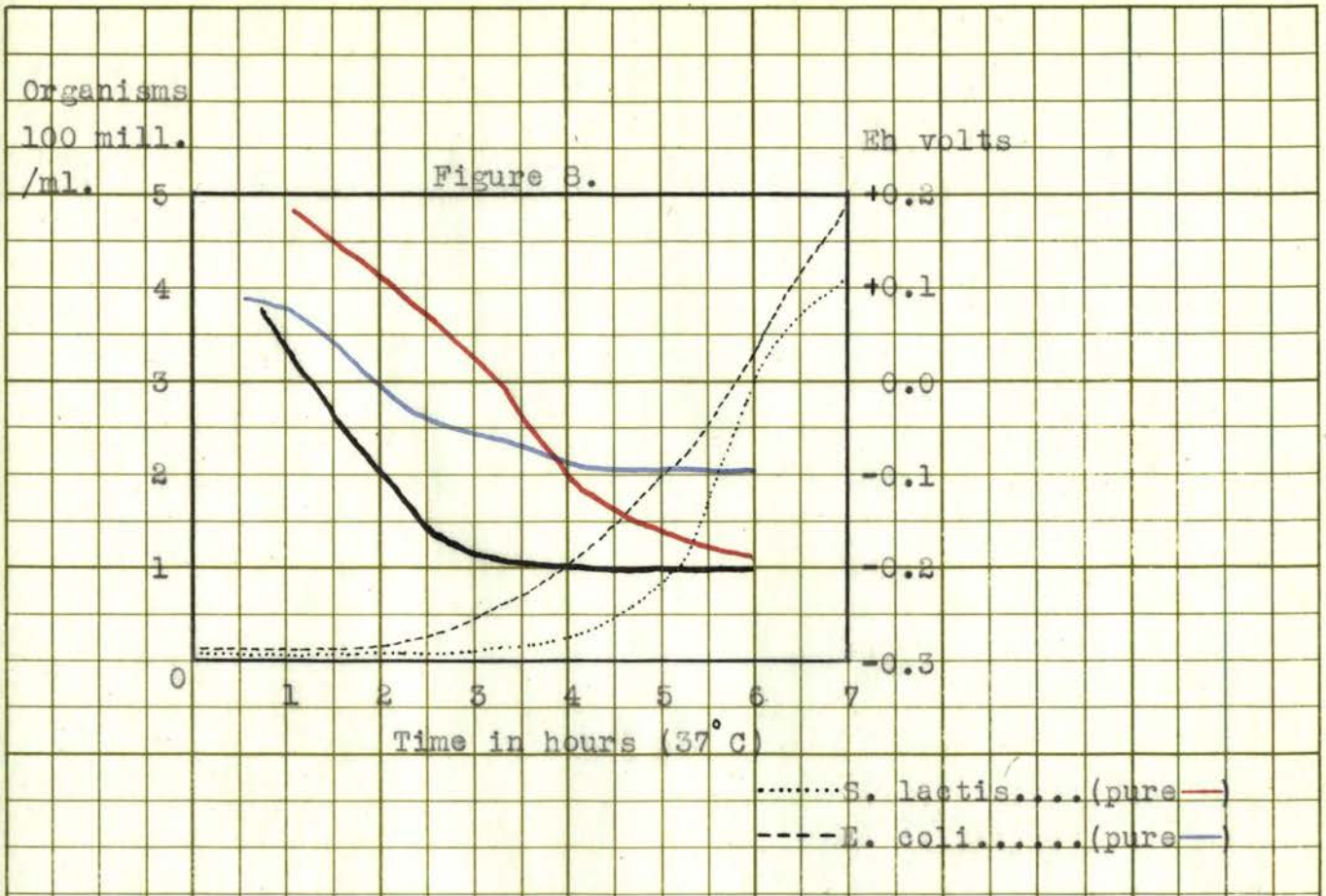
In their study of the effect of pure cultures on reduction time, Jones and Davis(12) found that S. lactis, S. agalactiae, S. liquefaciens, E. coli, A. aerogenes, Serratia marcescens, and Micrococcus roseus reduced methylene blue before resazurin. With cultures of other organisms, resazurin was reduced before methylene blue. Examples of this are L. acidophilus and B. subtilus. With Staphlococcus aureus the reduction of the two dyes always coincided. In practically every case the organism capable of souring milk reduced resazurin rapidly, the noticeable exceptions being S. agalactiae and B. subtilus which were slow reducers. Pure cultures vary markedly in their power to reduce resazurin but, generally speaking, a faster reduction of the dye is obtained with a more actively souring organism.

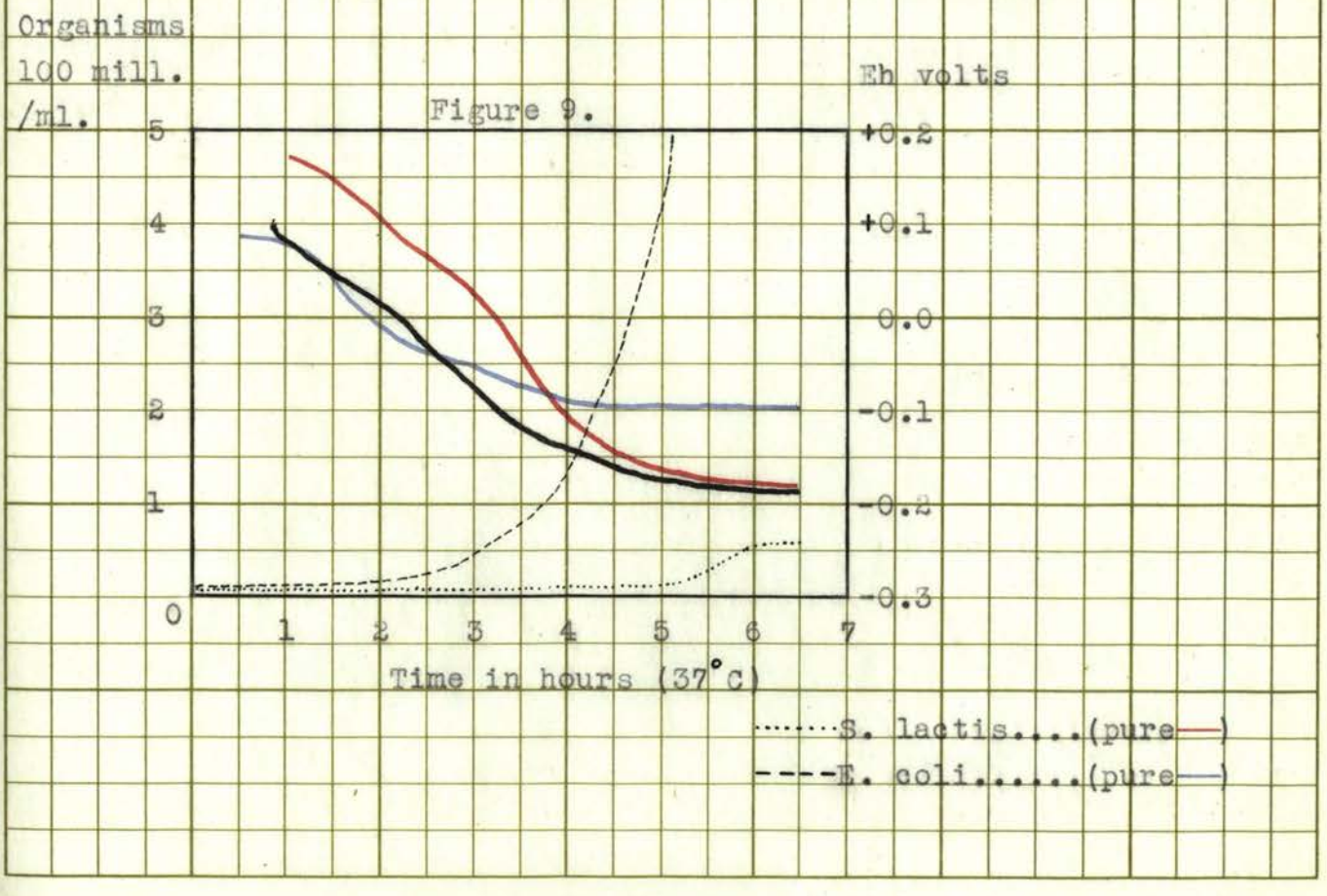
The influence of pure cultures of various milk bacteria on the oxidation-reduction potential of milk has been discussed. This influence would be important in dealing with samples of milk in which one of these organisms predominates or, in the extremely rare cases, where a pure culture of one organism

is present. In most samples of milk two or more species of bacteria are present in considerable numbers, and their associative growth may have an important effect both on the multiplication and on the oxidation-reduction potential of the milk. Changes in the oxidation-reduction potential of milk in which various pairs of cultures of milk bacteria were inoculated in different proportions were reported by Frazier and Whittier(7). Also, they recorded the changes in numbers of bacteria of the two species (see figures 8 to 11).

In figures 8 and 9 are shown results with S. lactis and E. coli inoculated into milk in different proportions and incubated at 37° C. When equal proportions (0.5 ml. inoculum) of cultures of S. lactis and E. coli were added to milk (fig. 8), both cultures grew well, but the Eh curve was more like that of S. lactis than that of E. coli. The curve flattened at about - 0.2 volt, instead of going down to - 0.22 volt, the figure characteristic for this strain of S. lactis. The Eh curve flattened before the beginning of the rapid rise in the growth curve of S. lactis, and after the rapid rise had started in the E. coli growth curve. In this case, then, the influence of S. lactis upon the oxidation-reduction potential was greater than that of E. coli, although the latter organism did exert a slight restraining influence.

When 1.0 ml. of E. coli culture and 0.1 ml. of S. lactis culture were added (fig. 9), the Eh curve was more that of E. coli, although after six hours the Eh values were still


Organisms
100 mill.
/ml.
Figure 8.
Eh volts
5
4
3
2
1
0
+0.2
+0.1
0.0
-0.1
-0.2
-0.3
1
2
3
4
5
6
7
Time in hours (37° C)
S. lactis....(pure)
E. coli.....(pure)


Organisms
100 mill.
/ml.
Figure 9.
Eh volts
5
4
3
2
1
0
+0.2
+0.1
0.0
-0.1
-0.2
-0.3
1
2
3
4
5
6
7
Time in hours (37° C)
S. lactis....(pure)
E. coli.....(pure)

dropping toward values characteristic of S. lactis. In a mixture of S. lactis and E. coli, then, S. lactis determines the final oxidation-reduction potential and, from the start, influences it more strongly than does E. coli.

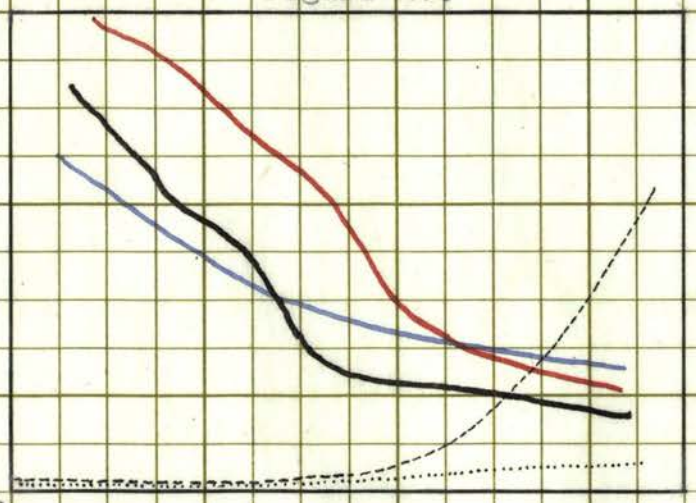
When an inoculum of 1 ml. each of S. lactis and A. aerogenes was used (fig.10), the coccus largely determined the changes in oxidation-reduction potential. From an examination of the Eh curves for pure cultures of these bacteria, it would seem probable that the combination of lactic organisms and gas-formers would hasten the drop in Eh to the value at which the Eh curve of a pure culture of the gas-former begins to flatten. From this point on, the colon-aerogenes organisms would tend to retard a further drop in oxidation-reduction potential to the value characteristic of S. lactis. Evidentaly, the larger the proportion of actively growing colon-aerogenes organisms, the greater is the restraining action.

In figure 11 S. fecalis apparently completely controlled the Eh changes when it was grown with L. bulgaricus, although both organisms grew well. In the same way S. thermophilus (not illustrated) dominates in Eh changes when grown with L. bulgaricus.

Experiments with B. subtilus and S. lactis, and with B. albolactis and S. lactis have shown that the proteolytic rods have little influence on changes in Eh and the curves are as if S. lactis alone were present(7).

The physiological types of bacteria found in milk have

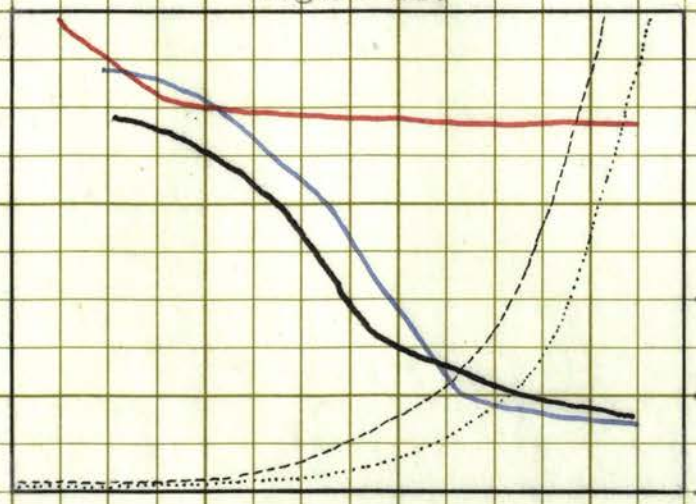
Figure 10.




-----S. lactis.....(pure——)

.....A. aerogenes....(pure——)

Figure 11.




----L. bulgaricus...(pure——)

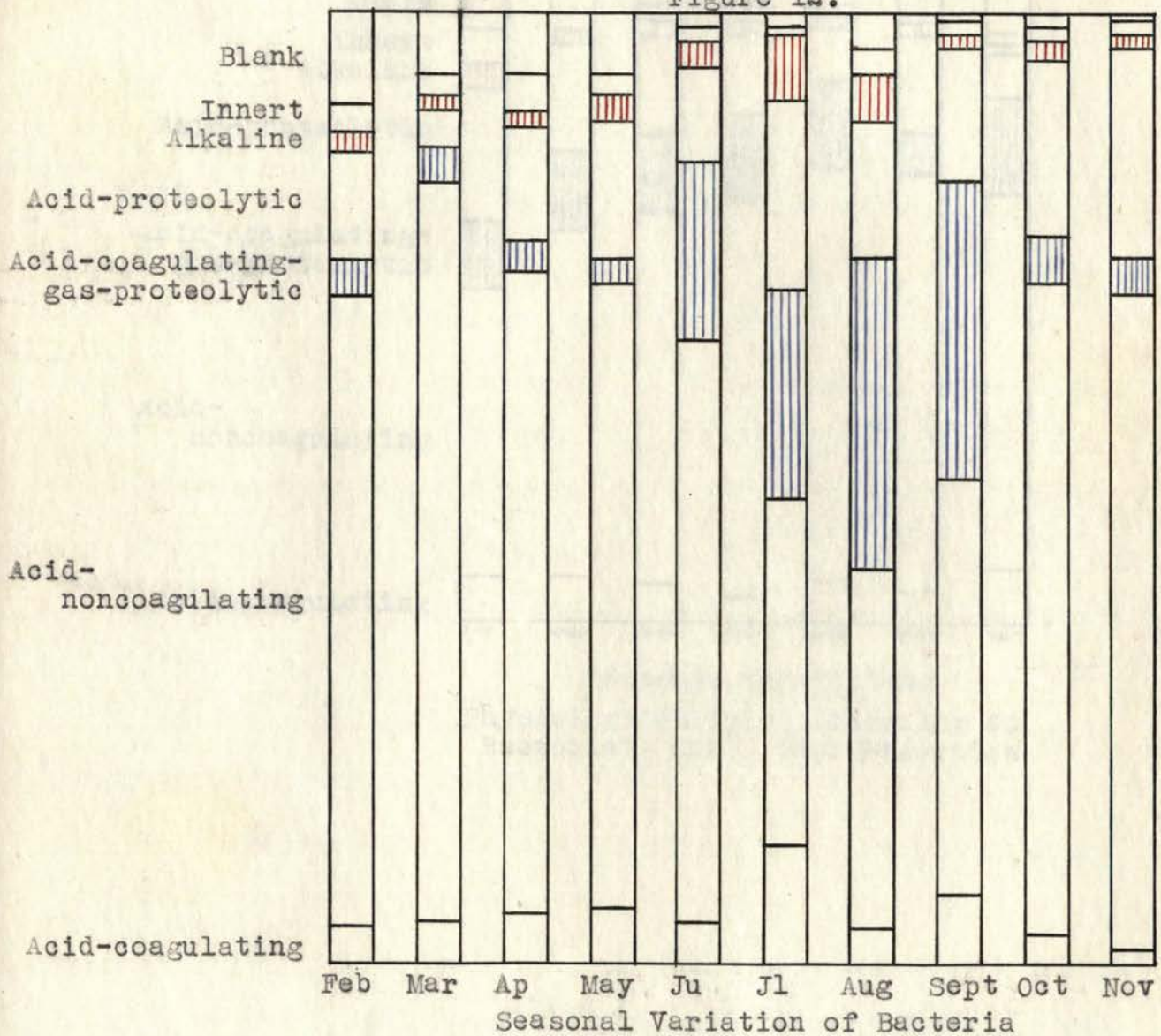
.....S. fecalis.....(pure——)

been determined by Davis and Lines(3) and they show the following relationships to the reduction of methylene blue. The average proportions of different types of bacteria in milk (determined by litmus milk) before a reduction test are 6.27 per cent acid-coagulating, 55.82 per cent acid-noncoagulating, 11.59 per cent acid-coagulating-gas-proteolytic, 16.56 percent acid-proteolytic, 2.68 per cent alkaline, 2.83 per cent inert, and 4.26 per cent blank (technique error or did not grow).

However, there are seasonal variations from the average proportion of types of bacteria in milk (see fig. 12). During the spring and summer months the number of acid-noncoagulating bacteria constantly decreased until fall. During these same months the numbers of acid-coagulating, alkaline and the acid-coagulating-gas-proteolytic types of bacteria increase. Other types vary considerably from month to month but the variation is not uniform. From September until March the variation in type is exactly opposite. This is thought to be significant in view of the fact that the average time of reduction decreases during the spring and summer and increases during the winter months, showing approximately the same trend as the acid-noncoagulating types and a trend exactly opposite to that shown by the acid-coagulating, alkaline and acid-coagulating-gas-proteolytic types.

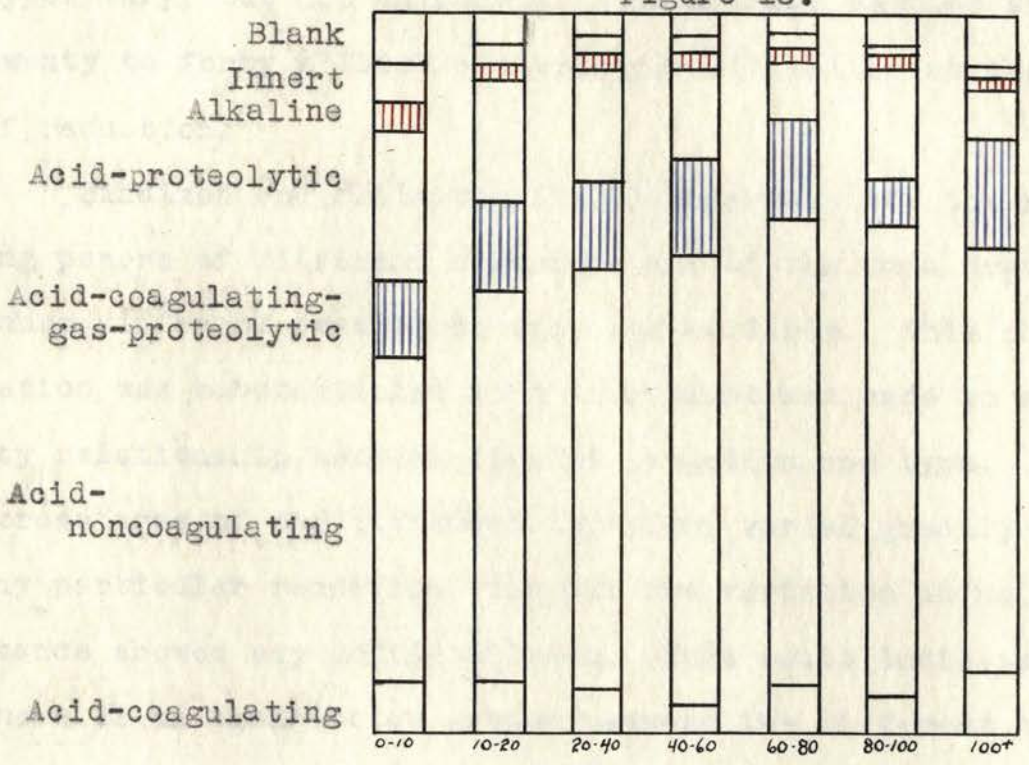
Figure 13 shows that as the plate count after reduction

Figure 12.




Seasonal Variation of Bacteria

Figure 13.




Bacteria in millions

Physiological Types According to Bacterial Count After Reduction

increases, the number of acid-coagulating-gas-proteolytic bacteria increases, as a rule, and the number of alkaline, inert and acid-proteolytic bacteria decrease. The other types vary, but not uniformly. The average samples show twenty to forty million bacteria per milliliter at the time of reduction.

Bindixen and Ellington (1932) observed that the reducing powers of different organisms and of the same organism under different conditions appeared variable. This observation was substantiated when an attempt was made to show any relationship between time of reduction and type. The percentages of the different organisms varied greatly at any particular reduction time but the variation in no instance showed any definite trend. This would indicate that there is an associative action between the different types of bacteria, and that the rate of oxygen absorption by the different types of bacteria is very materially influenced by their co-habitation with other types of bacteria(3).

In time-count relationships, the acid-noncoagulating type of bacteria showed a tendency to increase and the acid-proteolytic types trended to decrease as the count increased. This tendence was not constant. In a further study it was noted that, in the shorter reduction times, the acid-non-coagulating types were not numerous and that the acid-proteolytic types and acid-coagulating types predominated. Just the opposite of this was true in longer reduction times. On the basis of these time-count type studies, with the count

in thousands before reduction and in millions after reduction, in which the physiological types both before and after reduction also were considered, it was concluded that there is no very close relationship between physiological type and reduction time.

Table 2.

| Reduction time | Plate Count<br>Average count after reduction |
|---|---|
| 0 - 2 hrs. | 10 million/ml. |
| 2 - 3 " | 17 " |
| 3 - 4 " | 19 " |
| 4 - 5 " | 21 " |
| 5 - 6 " | 21 " |
| 6 - 7 " | 23 " |
| 7 - 8 " | 22 " |
| 8 - 9 " | 33 " |
| 9 - 10 " | 37 " |
| 10 - 11 " | 36 " |
| 11 - - " | 61 " |

A study of the relationship between the reduction time and the plate count before reduction, as well as the count after reduction, was made. It was found that there was no direct relationship between the count before and the count after reduction. There is a relationship between the original count and the time of reduction. As a general rule the time for reduction varies inversely with the original count, although there are numerous digressions from this rule (see table 2).

In table 2 it will be observed that, as the time for reduction increases the count increases. It shows clearly that one or more types present must have an inhibitory effect

on one or more of the other types, since the bacteria do not multiply at the even rate which would indicate independent growth.

Several conclusions may be drawn from the above information. The decrease in the number of acid-noncoagulating type of bacteria which followed the decrease in time of reduction would suggest that acid-noncoagulating types are slow reducers. On the other hand, during the same period in which the acid-noncoagulating types decreased there was an increase in the number of alkaline and acid-coagulating-gas-proteolytic types of bacteria. This would indicate that the alkaline and acid-coagulating-gas-proteolytic types are of primary importance in reduction. Acid-noncoagulating types predominate in good quality raw milk and so must play a large part in the reduction of methylene blue and resazurin. The acid-coagulating-gas-proteolytic types make up only 11.59 per cent of all bacteria in the samples of this study but must have played a rather significant role in reduction of the dyes and oxygen absorption. The small percent of alkaline types (2.83) percludes their being of much importance as regards reduction time.

Since the plate count of the bacteria increases after reduction as reduction time increases (table 2) it can be assumed that the bacteria in those samples having a high reduction time are rather inefficient reducers of methylene blue.

Since proteolytic types of bacteria have been shown to

have some special reducing power, it seems that the ability of the methylene blue test to indicate quality of milk is stregthened. Proteolytic types of bacteria in milk are particularly objectionable, especially from the standpoint of cheese products or other by-products which must be stored a long time(3).

It has been found difficult to estimate the true significance of the number of leucocytes and other types of cellular material in milk without knowledge of the reason for their presence. Leucocytes may be present in large numbers in apparently normal milk, so far as regular chemical and bacteriological examination shows. Duran-Jorda(5) believes that a number of fat droplets are carried to the mammary gland by cells; the cells responsible are a type of polymorphonuclear neutrophil which changes its nuclear morphology and its size before it undertakes the process of secretion. After secreting and expelling its cytoplasmic fat droplets the cell has the appearance of a lymphocyte. If this be the case, then the leucocytes present in the milk represent the wastage from the active mammary gland and have no sanitary significance. Grassi(8) found that cows show an average leucocyte count of 120,000/ml. The length of lactation period affects the leucocyte count since during the first month of lactation the number is low, and each following month the number is higher. Infection of the udder results in counts over 500,000/ml., the average being 950,000/ml. Breed(1) believes that if a large number of

leucocytes (500,000 or more), with polymorphonuclear types predominating, are accompanied by long chain streptococci, then the condition indicates, with pratical certainty, a streptococcic udder infection. Small numbers of leucocytes with no indication of accompanying bacterial infection indicate normal milk, so far as microscopic examination is concerned. Intermediate conditions are difficult to interpret. Other udder infections may cause an increased number of leucocytes to appear in the milk.

Thomas and Probert(14) found that increasing leucocyte counts were associated with a well defined increase in the rate of resazurin reduction, together with a similar but less marked increase in methylene blue reduction. Leucocyte counts under 500,000/ml. had very little influence on the reduction of either indicator. A leucocyte count between 500,000 and 2,000,000/ml. had an appreciable effect on resazurin, but no marked effect on methylene blue reduction. Leucocyte counts over 2,000,000/ml., however, had a strong reducing effect on both indicators.

Standard Methods(18) states:

> "Practically all methods of determining whether samples of milk or cream conform to the prescribed sanitary requirements have shortcomings of one kind or another, and this is especially true of methods for determining the bacterial content. Such limitations are not serious provided they are not ignored when interpreting results of tests. Since each method depends in part upon a different principle, the variables differ both in character and magnitude. Because each method is subject to these inherent and uncontrollable variables, both bacterial counts and reduction times should be regarded essentially as "estimates"

> and, furthermore, in the case of individual samples the results by one method should not be interpreted or reported in terms of results by another method. Implications that one method is in all cases indisputably more accurate than another are not valid".

Nevertheless the fundamental purpose of each of these tests is to determine whether a milk supply is satisfactory or unsatisfactory. A comparison of the results obtained by applying several tests to the same sample indicates the condition under which one test may replace another without significant changes in the results.

The agar plate method and the direct microscopic method are bacteriological tests normally employed on raw milk, along with the "reductase" tests. Correlations of these bacteriological tests have been made and Standard Methods(18) gives the following standards for those correlations for raw milk to be pasturized: (1) an average Standard Plate count/ml. for "Grade A" milk not to exceed 200,000, (2) an average Direct Microscopic Count of clumps/ml. for "Grade A" milk not to exceed 200,000, and (3) for the Methylene Blue Reduction Method for "Grade A" milk a reduction time of not less than six hours.

For the correlation of resazurin and methylene blue thiocyanate reduction tests on milk the following apparatus and materials were used:

(1) Dipper - As described in Standard Methods(18).

(2) Pipette - Delivery 10 ml. with 1.0 and 0.1 ml. graduations.

(3) Culture tubes - Screw cap (plastic), 20 by 150 mm., glass.

(4) Water bath - Inspissator operated at 37°C.

(5) Wire baskets - For holding tubes vertically in the inspissator and to keep the two tests separate.

(6) Light resistant flasks - Two amber bottles (400 ml.) for stock solutions of dyes.

(7) Methylene blue thiocyanate tablets - Dye content per tablet 9.1 mg., certified by the Biological Stain Commission.

(8) Resazurin tablets - Dye content per tablet 14.0 mg., certified by the Biological Stain Commission.

(9) Stock solutions of dyes - One tablet in 200 ml. of steril distilled water at pH 7.0.

(10) Apparatus and materials for plate counts and microscopic counts, as given in Standard Methods(18).

Standard Methods(18) was followed in the sterilization, make-up and use of the apparatus and materials, except in the places noted in the following discussion.

The samples of producer milk were collected by the sanitarians of the Wichita-Sedgwich County Health Department, at

four Wichita dairies, on routine sampling days, and the samples marked with the producer's number. The dipper was employed to transfer the milk from the weighing vat, or the milk can, to the culture tubes, and the tubes were filled with 25 to 30 ml. of milk. It was found that the dipper method is a satisfactory, practical and swift method of transferring milk to culture tubes, if adequate sanitary safeguards are provided. The procedure advocated in Standard Methods (18), using chlorine sterilization, is an adequate safeguard, but care must be taken to prevent milk from getting into the chlorine solution, because the fat content of the milk inactivates the chlorine. Note that the milk was not measured by the dipper method into 10 ml. portions at the time of collection. This was not done for two reasons: (1) extra milk was needed for the plate and microscopic tests, and (2) the use of a dipper as a measuring devise results in a large variation in the amount of milk obtained for each tube. The variation ranges from 1 to 11 ml. Such a variation affects not so much the dye concentration as the number of bacteria present in the sample, an important factor in determination of the rate at thich reduction will progress. Very large discrepancies (1 ml.) would maturally affect the grading of borderline milks. It was found that the quantity of milk tended to be below 10 ml., a condition that results in a reduction time longer than normally might be expected. For these reasons pipettes were used exclusively in measuring the quantities of milk at the laboratory. Screw cap

culture tubes were used because they offer little possibility of becoming contaminated by handling during the collection of the milk samples, or in setting up the tests. The hands or fingers of the technician do not come in proximity to the surfaces that are in contact with the milk in the tube. Screw cap culture tubes are readily adaptable to an aseptic technique that is practicable from the point of both the time and effort involved.

The samples were placed in an iced container and delivered to the University laboratory. At the laboratory, the samples were inverted fifteen times, and 10 ml. of milk was pipetted into a steril culture tube. Another 10 ml. of milk was transferred to a second culture tube. Each tube had recorded on it, the producer's number and the name of the dairy. One tube from each dairy then was placed in each of two baskets and refrigerated at 2° to 3° C. The remaining part of the sample was used immediately for plate and microscopic tests according to the procedure given in Standard Methods(18).

The next morning at 7:20 A.M., 1 ml. of methylene blue stock solution was added to each of the tubes in one of the baskets and put into the inspissator and protected from light. At 7:50 A.M., 1 ml. of resazurin stock solution was added to each of the tubes in the other basket, and placed in the inspissator. The tests were read every hour thereafter, until each dye was reduced to its end-point, the point at which an arbitrarily selected color appeared or for a period of eight

and one-half hours. The methylene blue end-point was considered to have been reached, when the milk was not less than four-fifth white. The color change of the resazurin samples was recorded hourly for the first 323 samples. The time required for reduction to the pink end-point was recorded for the next 353 samples. The color change of resazurin was determined by using a comparator chart (Nu-Comparator, Meyer-Blanke Company, St. Louis, Missouri). Daylight was used in reading the methylene blue test but fluorescent light brings out the color gradations of resazurin better than daylight. Exposure to extreme light sources was kept to a minimum, to avoid errors in the test. The resazurin samples were inverted slowly three times before they were read, to insure an even distribution of color in the milk. The methylene blue tubes were inverted the same number of times so that conditons might be comparable in both tests. The data from both tests were recorded under the producer's number, along with data from the plate or microscopic counts.

A total of 676 samples was obtained from 396 producers, a sampling which represents slightly less than half of the producers in the Wichita milk shed. The samples were picked at random, except that, in order to obtain a better correlation on the low grade milks, samples were taken more frequently from routes which had shown a poor quality of milk in the past. Therefore, the number of low grade samples in relation to the total number obtained is higher than normally could be expected.

Tables 3 to 5 show the color of the resazurin samples, and those containing methylene blue, at one, three and four hours. Two kinds of correlations may be observed. One correlation shows the total number of passing and non-passing resazurin samples, as compared with the methylene blue samples. The other correlation shows the number of qualitative agreements on individual samples as detected by resazurin and methylene blue.

Table 3.

| Color | Resazurin | Methylene blue | |
|---|---|---|---|
| | | 6 hrs/above | 5 hrs/below |
| Blue | 234 | 200 | 34 |
| Mauve | 77 | 56 | 21 |
| Purple | 5 | 1 | 4 |
| Pink/white | 7 | 0 | 7 |
| Total | 323 | 257 | 66 |

ONE HOUR TEST

From the results recorded in table 3, note that the selection of an end-point for resazurin, if based upon the results of the methylene blue test, would be limited to the mauve or purple colors, since the large majority of the resazurin samples fall in the blue to mauve range. The two possible selections for the end-point are given below:

(1) A choice of mauve as the end-point would result in the passing of 234 resazurin samples (89 non-passing), while 257 of the methylene blue samples are of passing grade (66 non-passing). With this end-point resazurin would pass 23 samples less than methylene, which give a correlation on the

total numbers of passing milks of 91.1 per cent. A disagreement on the quality of individual samples would be present in 91 samples (34 non-passing and 57 passing methylene blue samples) with a correlation of only 71.3 per cent.

(2) If purple be chosen as the end-point, 311 resazurin samples would pass (12 non-passing). This would result in a correlation on the total numbers of passing milks of 82.6 per cent. A disagreement on the quality of the individual samples would be present in 56 samples (55 non-passing and one passing methylene blue samples) with a correlation of 82.4 per cent. Thus, an end-point might be found, somewhere in between mauve and purple (using the Munsell color standards which show 16 degrees of color range from blue to pink) that would give a better correlation on the total number of passing milks. The low percentage of agreements on the individual quality which would be obtained, would not increase the accuracy. It would appear, therefore, that the one hour resazurin test does not qualify as a substitute for the standard methylene blue test.

Table 4.

| Color | Resazurin | Methylene blue | |
|---|---|---|---|
| | | 6 hrs/above | 5 hrs/below |
| Blue | 84 | 84 | 0 |
| Mauve | 140 | 127 | 13 |
| Purple | 66 | 42 | 24 |
| Pink/white | 33 | 4 | 29 |
| Total | 323 | 257 | 66 |

THREE HOUR TEST

From the results recorded in table 4, the end-point falls within the purple to pink range. The two possible selections for the end-point are given below:

(1) A choice of purple as the end-point, would result in the passing of 224 resazurin samples (99 non-passing), while 257 of the methylene blue samples are of passing grade (66 non-passing). With this end-point, resazurin would pass 33 samples less than methylene blue, with a correlation for the total number of passing milks of 87.2 per cent. A disagreement on the quality of individual samples would be present in 59 samples (13 non-passing and 46 passing methylene blue samples) with a correlation of 81.7 per cent.

(2) With pink as the end-point, 290 resazurin samples would pass (33 non-passing). This would result in a correlation for the total numbers of passing milks of 88.6 per cent. A disagreement on the quality of the individual samples would be present in 41 samples (37 non-passing and 4 passing methylene blue samples) with a correlation of 85.9 per cent. Although an end-point somewhere between purple and pink might yield a better correlation on the total number of passing milks, the percentage of agreements on individual samples would not be materially increased. Thus, the three hour resazurin test is slightly better than the one hour resazurin test, but still lacks the accuracy that would make it a good substitute for the standard methylene blue test.

Table 5.

| Color | Resazurin | Methylene blue | |
|---|---|---|---|
| | | 6 hrs/above | 5 hrs/below |
| Blue | 43 | 43 | 0 |
| Mauve | 115 | 114 | 1 |
| Purple | 99 | 87 | 12 |
| Pink/white | 66 | 13 | 53 |
| Total | 323 | 257 | 66 |

FOUR HOUR TEST

From the results recorded in table 5, if pink be chosen as the end-point of resazurin, then a correlation for the total numbers of passing resazurin and methylene blue samples would be 100%. A disagreement on the quality of individual samples would be present in 26 samples (13 non-passing and 13 passing methylene blue samples) with a correlation of 91.9 per cent. The results obtained on all 676 samples, when the four hour resazurin test was used, showed a correlation for the total number of passing milks of 98.9 per cent. A disagreement on the quality of the individual samples was present in 45 samples with a correlation of 93.3 per cent. The four hour resazurin test provides greater accuracy than the one hour or three hour tests, and would be an acceptable substitute for the standard methylene blue test.

Tables 6 and 7 show the correlation of the results of both the methylene blue and resazurin tests with the plate count.

Table 6.

| Resazurin | Plate count | |
|---|---|---|
| | Below 200,000/ml. | Above 200,000/ml. |
| Blue/Purple | 173 | 14 |
| Pink/white | 30 | 49 |
| Total | 203 | 63 |

FOUR HOUR TEST

Table 6 shows the correlation of the four hour resazurin test with the plate count. With pink as the end-point, 173 resazurin samples would be of passing grade, while 203 samples would pass on the plate count. This results in a correlation of 85.2 per cent. The agreement on the quality of the individual samples shows a correlation of 83.5 per cent.

Table 7.

| Methylene blue | Plate count | |
|---|---|---|
| | Below 200,000/ml. | Above 200,000/ml. |
| 6 hrs/above | 172 | 12 |
| 5 hrs/below | 31 | 51 |
| Total | 203 | 63 |

METHYLENE BLUE TEST

Table 7 shows that the correlation of the plate count with the results of the methylene blue test is practically identical with those obtained with the resazurin test. When the plate count is compared with both reduction tests at the same time, it is found that the plate count agrees with the results of both reduction tests in 77.1 per cent of the total number of samples; it agrees with the resazurin results in 3.6 per cent of the samples, and with the methylene blue results in 3.4 per cent of the samples. It agrees with neither

reduction test in 15.9 per cent of the samples. The correlation obtained from the plate count and reduction tests indicates that, while the reduction tests agree very closely on the quality of the milk, there is little agreement between them and the plate count. Therefore, the plate count, as specified in Standard Methods(18), would not be a good criterion for judging the results obtained with either reduction test as applied to individual samples.

An interesting sidelight came to view, during the summer of 1950, when two species of the blue pigment producing genus Pseudomonas, were isolated. One of these species was found to be Pseudomonas aeruginosa and the other is believed to be a plant pathogen. The isolation of P. aeruginosa is of special interest in this area because of a recent endemic mastitis, due to infection with P. aeroginosa, in dairy herds at Great Bend, Kansas. As P. aeruginosa also is pathogenic to man, the isolation of this bacterium, from milk, should be of concern in determining the potability of milk. Fortunately, pasturization would eliminate this organism. The producer would be concerned directly because of the lowered quality of the milk, and of the infection of his cows.

The microscopic count shows about the same correlations with the reduction tests as the plate count, but its greatest asset is its ability to indicate the causal agent in those milks showing a short reduction time. It was found that microscopic examination of the milk immediately after reduction could usually detect one or more of the following causes of

the short reduction time: (1) the presence of leucocytes in abnormal numbers, or (2) the presence of a particular morphological type of bacterium. Microscopic examination of milks, showing a low reduction time, is a valuable supplementary test when the source, as well as the isolation and study, of the causal agents is of prime interest. Microscopic examination of samples with low reduction times reveals that lactic acid bacteria are the most numerous bacteria in milk; bacillary types are not frequently encountered. Long chain streptococci are rarely found but, when found, they are accompanied by large numbers (one million or more) of leucocytes. These conditions are typical of the mastitis caused by S. agalactiae. The rarity of these findings probably was due to the fact that samples were obtained from herd milk, rather than from individual cows. Herd milk dilutes abnormal milk to the extent that it is difficult to recognize its presence in a pooled sample. Therefore, any sample of herd milk which shows a heavy contamination of long chain streptococci and leucocytes probably indicates a high incidence of mastitis in the herd.

From the above results of tests on 676 samples of producer milk, several conclusions may be made:

(1) Milk samples should be measured with a pipette, rather than with a dipper.

(2) The plate count does not show a high degree of correlation with either the resazurin or methylene blue reduction tests, and thus, is limited in its use as a control for the reduction tests to very good or very bad quality milks.

(3) The four hour resazurin test, using the pink color as the end-point, is a better test than the one hour or three hour resazurin tests if it is to be used as a substitute for the methylene blue test.

(4) Resazurin results are obtained in a shorter time than are the results of the methylene blue test.

(5) Milk, showing a low reduction time, should be examined microscopically in order to ascertain the causal contaminant.

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