The Methylene Blue Reduction Test

A Thesis

Presented in partial fulfilment of the requirements for the

Degree of Master of Science in Agriculture in the

Department of Dairying

of the

University of Idaho

by

Irwin John Bibby, B. S.

1913

The Methylene Blue Reduction Test.

Investigators, who have studied the Methylene Blue Reduction Test, within the last few years, have reached somewhat different conclusions regarding the reliability of this test in determining the number of bacteria in milk.

As there are no accepted tests for determining the number of bacteria in milk, at present, in a period shorter than twenty-four hours, it is quite evident that a test like the Methylene Blue Reduction Test, which gives results in a few minutes or a few hours, should be of great value to creameries, cheese factories, and milk depots. The short time required to conduct this test and the ease with which it can be conducted are the promising features. The question that this thesis attempts to answer is, "Is the time required for reduction an index of the number of bacteria and of the kinds of bacteria in the milk, or does acidity, enzymes, or some other factor influence the time of reduction?"

Practically all of the investigators argue that the reduction runs parallel with the number of bacteria present in the milk. In other words, the greater the number of bacteria present, the shorter the time required for reduction. The greatest difficulty with the test seems to be in determining a standard length of time, which is required by a given number of bacteria, to reduce the methylene blue. The results of different investigators vary on this particular point.

History

The first work on the reduction test was conducted by Duclaux (1) in 1887. His method was somewhat different

from the method used at the present time. Yet the present methylene blue test probably originated from this beginning. Instead of using methylene blue for coloring the milk, Duclaux used indigo carmine. His conclusions were that the reduction was caused by ferments.

No further investigations were reported along this line of work until 1900. At this time Neisser and Nechberg (2) conducted experiments in which they used methylene blue for coloring the milk. Their conclusions were that the reduction was caused by living micro-organisms. In 1901 Ninter Blythe (3) published an article on the reduction test in which he used litmus for coloring the milk. He did not arrive at any definite conclusion as to the cause of the reduction process.

Schardinger (4) in 1902 published a report on experiments which he had conducted on the reduction power of milk. His experiments differed somewhat from those previously published. He used a solution of methylene blue and formalin for coloring the milk. It was prepared as follows:

Alcoholic Sol. Meth. Blue ..... 5 c.c.

Formalin ..... 5 c. c.

Distilled Water .....190 c.c.

In his conclusion he states that no doubt the reduction of methylene blue in milk may be a very important test for household purposes, providing the reduction has a definite strength in accordance with the number of bacteria in milk. He also states that a slow reduction indicates the freshness of the milk and also a small number of bacteria. The acidity

and the time of reduction, he concludes, bear no definite relation to each other.

Cathcart and Hahn (5) found also that the length of time required for the reduction to take place depended upon the number of bacteria present in the milk.

Orla Jensen (6) conducted experiments in 1903, in which he tested the reduction power of a number of organisms separately. He concluded that the lactic organisms required a longer time to reduce and the reduction was not as complete as with non-acid producing organisms..

In 1904 Neisser and Smidt (7) suggested that the power of reduction was due to one of the following factors:

(1) Milk sugar and substances that are separated by cooking.

(2) Reducing ferments.

(3) Reduction properties of bacteria.

They finally decided that the reduction was caused by an enzyme called aldehyde-catalase. In his later work Smidt (8) confirms his early investigations. He says that the reduction of the Methylene formalin solution is caused by an enzyme which acts as a catalytic agent, but the reduction of a Methylene blue solution without the formalin is caused entirely by bacteria.

Rullman (9) in 1904 and Koning (10) in 1907, found but very little reduction power in goats' milk and practically none in human milk unless it was diseased.

In experiments conducted by Seligmann (11) in 1906, he concluded that there was no difference between the reduction of Methylene formalin solution and methylene blue. He, also, concluded that the reduction properties of milk come from bacteria and by-products of milk casein. In order to reduce mehtylene blue or methylene formalin solution he decided that it must be a very c mplex solution. Seligmann was alone (on this idea) for some time.

Jensen (12) and Smidt (12) published a paper in which they agreed that the aldehydecatalase was bound up in the fat globules of milk and that the aldehydereductase was bound to the slime membrane. They were not successful in proving this, however.

In the meantime Seligmann (13) had continued his experiment and in 1907 his report confirmed his previous work.

A. Hesse (14) published a report in which he questioned some of the points of Schardinger's test and was somewhat skeptical as to the proof of the test. He did not draw any further conclusions.

Barthell (15), a Swedish investigator, published a paper in which he reported the results of his experiments with the methylene blue reduction test. He conducted experiments upon a much larger scale than any of the previous investigators. His method was as follows: To 10 c.c. of milk .5 c.c. of the methylene blue solution was added. The mixture was covered with 1 c.c. of liquid paraffin. The test tube containing the milk and methylene blue solution was then placed in a water bath and kept at a temperature of from 40 to 48 degrees C.. The length of time required for the discoloration

was noted. Two tests of the same milk were always conducted The object of his experiments was to determine the length of time required for the reduction of the methylene blue in milk by different numbers of bacteria. These relations had already been shown to exist by Buttenberg (16) and others, but had not been definitely proven. Barthell found that if the methylene blue was reduced in a few minutes the milk contained at least 100,000,000 bacteria or more per cubic centimeter. In cases in which the reduction took place within one hour, the milk was found to contain altogether too high a bacterial content to be used as a food, especially for infants. Milk which reduced the Methylene blue within three hours was found also to be of rather an inferior quality. If it required more than three hours for the milk to reduce the methylene blue the milk was found to be low in bacterial count, and of good quality. Barthell also found that with pasteurized milk the reduction proceeds more or less slowly according to the temperature at which the milk had been pasteurized. In conclusion Barthell decided that the reduction test can, no doubt, be used as a fundamental test for determining the bacterial content of milk. He states, also, that it is a very practical test, as it can be concluded within a few hours so that the quality of the milk can be determined before it is sent to the consumer.

About this time R. Burri and J. Kursteiner (17) conducted a number of experiments in regard to the reduction power of cow's milk as shown by the methylene blue reaction with and without formalin. They found that normal fresh milk did not

increase the reducing substance and if oxygen was excluded the reduction took place more quickly when the temperature was increased.

In a paper relating to studies of milk enzymes, C.J. Koning (18) reports that bacteria, which are generally present in milk, produce more or less reductase when they are introduced into sterile milk. He also decided that the reductase of milk is not destroyed when the milk is heated 30 minutes at 65 degrees C. and that the fat and cream of normal milk contains more reductase than skim milk.

R. Tronasdorff (19) in 1909 concluded from his experiments that fresh milk, which was germ free, contained no reductase. It gave, however, a characteristic reaction to the Methylene-formalin solution. He concluded that the formalin has a chemical action upon Methylene blue.

After reviewing the work of previous investigators, and condutcting some experiments of their own, R. Burri and J. Kunsteiner (20) in a paper published in 1912, arrived at somewhat different conclusions from former investigators. They decided that a normal raw milk contained reductase which was not of bacterial origin. They decided, however, that it might have some relation to the cellular elements, which were present, such as leucocytes and epithelial cells. It was found also that the quality of the Methylene blue might cause a difference in results. They decided that in all probability the reduction of Schardinger's methylene formalin solution was caused by an enzyme.

Probably the latest and most thoro work along this line is contained in a report by E. B. Fred (21) published in 1912. He found that Methylene blue was the most useful stain for measuring reduction by micro-organisms. It was decolorized more rapidly in milk than in bouillon. He found also that reduction was a general property possessed by all bacteria, but that it varies with different organisms. The bacteria of milk, however, show a strong reducing power. Out of twenty-two species generally prevalent in milk, twenty-one proved to be stain reducers. Reduction in a newly inoculated culture medium was directly proportional to the growth of bacteria, and stopped when the medium was exhausted. He decided that the ferment peroxidase is presen t in milk when secreted and is not formed to any extent by the growth of bacteria. The reductases, however, are formed by the growth of bacteria and do not occur in milk when first drawn. Very probably both intracellular and extracellular products take part in the reduction of the Methylene blue. The reduction of Schardinger's methylene-formalin solution is due to an enzyme known as Aldehydcatalase. Finally he concludes that the reduction test is of practical importance in judging the quality of milk from a bacteriological standpoint.

In reviewing the work of different investigators it was found that their conclusions vary somewhat, but that there is a general agreement. The prin cipal difference of opinion was over the best indicator to use in the reduction test, and also whether or not reduction was caused by

enzymes formed in milk, by the growth of bacteria, or by enzymes formed by agents other than bacteria. Various indicators were tried, but in most cases methylene blue was found to be the most useful in the test. There seemed to be a general opinion that the methylene-formalin solution is not reduced by bacteria, but by a ferment, which is present in the milk when drawn, and not formed by the growth of bacteria. It is called Aldehydcatalase. The methylene blue solution without the formalin was thought to be reduced by a ferment known as reductase and formed by the growth of bacteria. The length of time required for reduction to take place by certain number of bacteria was found by the different investigators to be about the same. The matter of time was estimated only roughly and definite standards were not very closely worked out.

Object

The object of this thesis is First: to determine the relation existing between the bacterial content and the time required for the reduction of methylene bluein milk; Second: to determine some of the common factors which favor or hinder the reduction test; and Third: to find out the practical value of the test for determining the bacterial content of milk.

Methods

The apparatus used in carrying on these experiments was constructed by the C. J. Tagliabue Manufacturing Company of New York City, especially for the reduction test. This apparatus was furnished us by theabove company without

charge. The apparatus consisted of a water bath, test tubes of 30 c.c. capacity, and a special thermometer. The methylene blue used for the experiments was prepared as follows (22):

Sat. Alcoholic Sol. Meth. blue ..... 6 c.c.

Caustic potash, 1 per cent solution ..... 2 c.c.

Water, distilled ;;.....; 20 c.c.

The gelatin used in making the bacterial count was as follows (23):

Water, distilled .....1000 c.c.

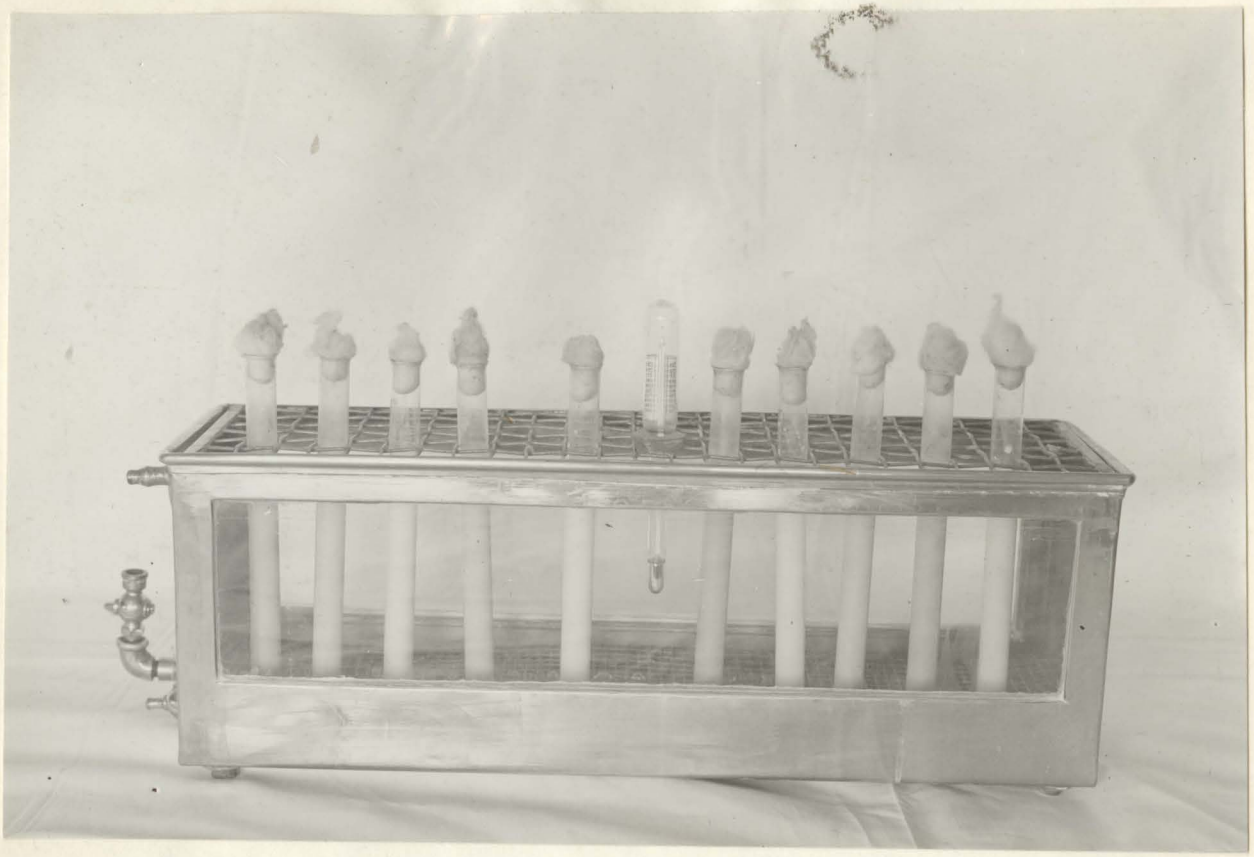
Peptone ..... 13 gms.

Gelatin ..... 150 "

Milk sugar ..... 30 "

Liebig's extract of beef .....7 "

Neutralized to .1 per cent acidity



Apparatus used for the methylene blue reduction test.

All milk samples were taken at the University Creamery as soon as the milk was delivered by the patrons. The temperature at which these experiments were conducted varied from 40 degrees to 45 degrees C. In one or two cases the temperature differed from this.

Experiment I.

This experiment was conducted in order to determine the length of time required for the complete reduction of methylene blue in milk. It was conducted also to determine whether or not the same number of bacteria always required the same length of time for reduction. In each case whole milk was used. Twenty cubic centimeters of the milk was placed in one of the sterile test tubes and two tenths of a cubic centimeter of the methylene blue solution added. Then the tube was stopped with sterile cotton and agitated until the methylene blue was evenly distributed thru the milk, giving it a light blue color. The tubes were prepared in this manner for each part of the experiment, then were placed in the water bath which was kept at a temperature of forty to forty-five degrees c.c. All of the tubes were run in duplicate, but they checked out so closely that only one result is given in each case. At the same time that the reduction test was started, samples of the milk were plated upon gelatin, incubated at 18 degrees C. and as soon as the plates were sufficiently developed counts were made. The reduction was considered complete when the milk assumed its natural color. The length

of time required for the reduction was noted in each case. All parts of this experiment were conducted in the same manner, but were not run on the same date. No attempt was made to get samples of the same number, in the different parts of this experiment, from the same dairy farms.

Experiment I Part I

Milk Sample Number	Length of time required	Number of Bacteria per c.c.
1	7 hrs. 10 min.	30,000
2	8 hrs.	150,000
3	7 hrs.	100,000
4	8 hrs. 30 min.	70,000

Experiment I Part II

Milk Sample Number	Length of time required	No. Bacteria per c.c.
1	8 hrs. 5 min.	90,000
2	7 hrs. 45 min.	100,000
3	7 hrs. 30 min.	78,000
4	1 hr. 50 min.	500,000

Experiment I Prt. 3

Milk Sample No.	Length of time required for reduction	No. Bacteria per c.c.
1	6 hrs.	100,000
2	5 hrs. 30 min.	120,000
3	1 hr. 5 min.	1,250,000
4	1 hr. 30 min.	600,000

Experiment Part 4

Milk Sample No.	Length of time required for reduction	No. Bacteria per c.c.
1	3 hrs. 10 min	200,000
2	3 hrs.	150,000
3	1 hr. 5 min.	300,000
4	1 hr. 25 min.	400,000

Experiment I Part 5

Milk Sample No.	Length of time required for reduction	No. Bacteria per c.c.
1	1 hr. 10 min.	14,000,000
2	1 hr. 30 min.	950,000
3	20 min.	4,000,000
4	27 min.	2,000,000

Experiment I Part 6

Milk Sample No.	Length of time required for reduction	No. Bacteria per c.c.
1	2 hrs. 15 min.	700,000
2	2 hrs. 55 min.	400,000
3	2 hrs. 30 min.	500,000
4	3 hrs.	350,000

Experiment I Part 7 (See note)

Milk Sample No.	Length of time required for reduction	No. Bacteria per c.c.
1	8 hrs. 30 min.	20,000
2	8 hrs. 15 min.	45,000
3	9 hrs. 11 min.	15,000
4	9 hrs. 30 min.	18,000

Note - This milk was pasteurized.

Experiment I Part 8

Milk Sample No.	Length of time required for reduction	No. Bacteria per c.c.
1	13 min.	20,000,000
2	10 min.	25,000,000
3	1 hr. 55 min.	600,000
4	1 hr. 5 min.	2,500,000

Experiment I Part9

Milk Sample No.	Length of time required for reduction	No. Bacteria per c.c.
1	2 hrs. 20 min.	420,000
2	2 hrs. 41 min.	380,000
3	2 hrs. 6 min.	560,000
4	1 hr. 12 min.	1,120,000

Experiment I Part 10

Milk Sample No.	Length of time required for reduction	No. Bacteria per c.c.
1	5 hrs. 55 min.	175,000
2	2 hrs. 53 min.	270,000
3	1 hr. 35 min.	900,000
4	2 hrs. 5 min.	500,000

Experiment I Part 11

Milk Sample No.	Length of time required for reduction	No. Bacteria per c.c.
1	1 hr. 35 min.	1,500,000
2	1 hr. 57 min.	700,000
3	2 hrs. 20 min.	350,000
4	1 hr. 13 min.	1,200,000

Experiment I Part 12

Milk Sample No.	Length of time required for reduction	No; Bacteria per c.c.
1	2 hrs. 45 min	190,000
2	3 hrs. 10 min.	200,000
3	2 hrs. 15 min.	410,000
4	1 hr. 45 min.	1,000,000

Experiment I Part 13

Milk Sample No.	Length of time required for reduction	No. Bacteria per c.c.
1	1 hr. 10 min.	1,500,000
2	1 hr. 45 min.	700,000
3	57 min.	1,750,000
4	2 hrs. 10 min.	400,000

Experiment I Part 14

Milk Sample No.	Length of time required for reduction	No. Bacteria per c.c.
1	7 hrs. 12 min.	90,000
2	5 hrs. 56 min.	120,000
3	2 hrs. 15 min.	320,000
4	3 hrs. 12 min.	150,000

Experiment I Part 15

Milk Sample No.	Length of time required for reduction	No. Bacteria per c.c.
1	4 hrs. 10 min.	180,000
2	3 hrs. 15 min.	270,000
3	2 hrs. 6 mn.	320,000
4	3 hrs. 25 min.	250,000

The results of the above experiment show, quite conclusively, that the time of reduction runs nearly parallel with the number of bacteria present in the milk. The test varied somewhat but the length of timerequired for reduction was about the same for any given number of bacteria. The results show that the test is accurate to within fifty or one hundred thousand bacteria per cubic centimeter as long as the bacterial count is below one million. When the count is greater than one million per cubic centimeter the variation is greater. In preparing a reduction table which will show the direct relation between the time required for the reduction to take place and the number of bacteria in the milk, the time cannot be estimated closer than thirty minutes in each case. The reason for this is because of the variation in the results of the previous experiment.

The following reduction table has been evolved from the results of the preceding experiment:

Time required for the reduction	No. Bacteria per c.c. in milk
10 min.	25,000,000
13 min.	20,000,000
20 min.	4,000,000
30 min.	3,000,000
1 hr.	2,000,000
1 hr. 30 min.	1,000,000
2 hr.	900,000
2 hr. 30 min.	600,000
3 hrs.	400,000
3 hrs.30 min.	300,000
4 hrs.	250,000
4 hrs. 30 min.	200,000
5 hrs.	150,000
6 hrs.	100,000
7 hrs.	80,000
8 hrs.	50,000
9 hrs.	20,000

In conducting the reduction test the results may vary from the above table, but with the majority of cases the table will be found to be accurate enough for all practical purposes.

Experiment II

This experiment was conducted in order to determine whether or not the acidity of the milk had any influence upon the length of time required for reduction. Also, to determine what effect the different groups of organisms, such as acid producers, and liquefiers had upon the reduction test. The number of acid formers was determined by inoculating tubes of litmus milk with different dilutions of milk. The liquefiers and non-liquefiers were determined by plating samples of the milk on the gelatin previously described. The results were as follows:

Experiment II Part 1

Sample No.	1	2	3	4
Acidity	14%	18%	16%	15%
Liquefiers	7000	2000	3000	40000
Lactic organisms	10000	50000	10000	100000
Non-Acid Prod. or Liquefiers	68000	48000	37000	360000
Total No. Bacteria	95000	100000	70000	500000
Time of reduction	8hrs.40min	7 hr30mn	8hr20min	6 hrs.

Experiment II Part 2

Sample No.	1	2	3	4
Acidity	19%	18%	15%	16%
Liquefiers	600	2000	3000	1200
Lactic organisms	10000	10000	10000	50000
Non-Acid Prod. or Liquefiers	14000	58000	37000	38000
Total number of bacteria	30000	70000	40000	100000
Time of reduction	7 hrs.	6 hrs. 30 min	8 hr. 20 min.	9 hrs. 10 min.

Experiment II Part 3

Sample No.	1	2	3	4
Acidity	15%	16%	15%	17%
Liquefiers	12000	30000	40000	20000
Lactic organisms	50000	100000	100000	100000
Non-Acid Prod. or Liquefiers	28000	870000	750000	400000
Total No. Bacteria	90000	1000000	1250000	700000
Time of reduction	6 hrs.	5 hr. 30 min.	1hr.15min.	1hr.30min.

Experiment II Part 4

Sample No.	1	2	3	4
Acidity	21%	23%	18%	17%
Liquefiers	120000	130000	40000	50000
Lactic organisms	100000	500000	100000	100000
Non-Acid Prod. or Liquefiers	----	----	160000	260000
Total NO.Bacteria	200000	250000	300000	400000
Time of reduction	3 hrs. 30 min.	3hr.20mn.	55 min.	1hr.20min

Experiment II Part 5

Sample No.	1	2	3	4
Acidity	20%	23%	25%	21%
Liquefiers	2500000	3350000	3000000	2500000
Lactic Organism	1000000	5000000	10000000	10000000
Non-Acid Prod; or Liquefiers	9700000	650000	17000000	8500000
Total NO.Bacteria	13000000	9000000	30000000	20000000
Time of reduction	1 hr. 10 min.	1 hr. 30 min.	20 min.	27 min

Experiment II Part 6

Sample No.	1	2	3	4
Acidity	21%	19%	24%	17%
Liquefiers	600000	50,000	200,000	150,000
Lactic Organisms	500,000	300,000	1,000,000	300,000
Non-Acid Prod. or Liquefiers	500,000	650,000	700,000	400,000
Total No.Bacteria	1,600,000	1,000,000	1,900,000	850,000
Time of reduction	1hr. 5min.	1hr. 37min.	57 min.	2hrs.25min

Experiment II Part 7

Sample No.	1	2	3	4
Acidity	.28	.36	.30	.29
Liquefiers	1,000,000	1,200,000	1,500,000	2,000,000
Lactic Organisms	1,000,000	1,500,000	1,000,000	10,000,000
Non-Acid Prod. or Liquefiers	1,600,000	----	2,500,000	2,000,000
Total No. Bacteria	3,600,000	2,600,000	4,000,000	14,000,000
Time of reduction	1 hr.15 min.	1 hr.40 min	1 hr.30 min	25 min.

Experiment II Part 8

Sample Number	1	2	3	4
Acidity	.21	.18	.23	.26
Liquefiers	1,800,000	900,000	1,500,000	2,000,000
Lactic Organ-isms	500,000	500,000	1,000,000	1,500,000
Non-Acid Prod. or Liquefiers	200,000	300,000	300,000	100,000
Total No. Bacteria	2,500,000	1,500,000	2,800,000	3,600,000
Time of reduction	46 min.	56 min.	41 min.	37 min.

Experiment II Part 9

Sample Number	1	2	3	4
Acidity	16%	14%	13%	11%
Liquefiers	46,000	75,000	100,000	88,000
Lactic Organisms	200,000	150,000	50,000	50,000
Non-Acid Prod. or Liquefiers	554,000	400,000	150,000	250,000
Total No. Bacteria	800,000	625,000	300,000	385,000
Time of reduction	2 hrs.30 min	3 hrs.10 min	4 hrs. 5min	4 hrs. 15 min.

Experiment II Part 10.

Sample Number	1	2	3	4
Acidity	12%	10%	14%	13%
Liquefiers	40,000	20,000	85,000	90,000
Lactic Organisms	200,000	100,000	150,000	200,000
Non-Acid Producers	175,000	150,000	165,000	85,000
Total No. Bacteria	425,000	275,000	400,000	375,000
Time of reduction	2 hrs.50min.	3 hrs.55"	3hrs.10"	3hrs. 45"

Altho the results of the above experiment varied considerably it is quite evident that the acid producing organisms did not influence the process of reduction as much as the non-acid producing bacteria. The liquefying bacteria did not shorten the time of reduction any more than an equal number of non-liquefying bacteria. The degree of acidity did not have a noticeable effect upon the time of reduction.

Experiment III

The previous experiments raised the question as to whether or not all organisms found in milk have the power of reducing methylene blue. This experiment was conducted with sterile milk inoculated with pure cultures. Each of the tubes of milk were inoculated with a loop full of the

organism to be tested and incubated at 28 degrees C. for a period of twenty-four hours before c nducting the test. Two tests were conducted with each organism on separate days. Four tubes of inoculated milk were used in each test and a tube of sterile milk, uninoculated, was used as a check in each part of the experiment. The same amount of methylene blue was added as in the previous experiments, and each tube contained 20 c.c. of milk. The results were as follows:

Experiment III Part 1

B. Subtilis

Sample Number	1	2	3	4	5
Time to reduce	40 min.	45 min.	38 min.	54 min.	No reduction
No. Bacteria	410,000	350,000	385,000	300,000	0
Complete reduction	Plus	Plus	Plus	Plus	Minus

Sample Number	1	2	3	4	5
Time to reduce	1 hr. 5"	35"	42"	50"	No reduction
No.Bacteria	110,000	425,000	350,000	310,000	0
Complete reduction	Plus	Plus	Plus	Plus	Minus

Experiment III Part 2.

B. Coli

Sample Number	1	2	3	4	5
Time to reduce	15 min.	17 min.	20 min.	25 min.	No reduction
No. Bacteria	30,000,000	28,000,000	24,000,000	17,000,000	0
Complete Reduction	Plus	Plus	Plus	Plus	Minus

Sample No.	1	2	3	4	5
Time to reduce	24 min	19 min.	15 min.	17 min.	No reduction
No. Bacteria	21,000,000	30,000,000	35,000,000	38,000,000	0
Complete Reduction	Plus	Plus	Plus	Plus	Minus

Experiment III Part 3.

B. Yeast (Bread)

Sample No.	1	2	3	4	5
Time to reduce	5 min.	8 min.	10 min.	6 min.	No reduction
No. Bacteria	4,500,000	3,800,000	3,200,000	4,000,000	0
Complete Reduction	Plus	Plus	Plus	Plus	Minus

Sample No.	1	2	3	4	5
Time to Reduce	12 min.	9 min.	15 min.	6 min.	No reduction
No. Bacteria	2,400,000	3,500,000	2,500,000	5,000,000	0
Complete Reduc.	Plus	Plus	Plus	Plus	Minus

Experiment III Part 4

Bact. Acidi Lactici

Sample No.	1	2	3	4	5
Time to reduce	25 min.	45 min.	55 min.	1 hr. 10"	Not reduced
Bacteria No.	1000,000,000	550,000,000	450,000,000	235,000,-000	0
Complete reduction	Minus	Minus	Minus	Minus	Minus

Sample No.	1	2	3	4	5
Time to reduce	35 min.	40 min.	50 min.	27 min.	Not reduced
Bacteria No.	600,000,000	530,000,000	350,000,000	1,260,-000,000	0
Complete reduction	Minus	Minus	Minus	Minus	

Experiment III, Part 5

Bacillus Vulgaris

Sample No.	1	2	3	4	5
Time to reduce	20 min.	25 min.	19 min.	36 min.	Not reduced
Bacteria No.	15,000,000	11,000,000	13,500,000	5,500,000	0
Comp. Reduc.	Plus	Plus	Plus	Plus	

Sample No.	1	2	3	4	5
Time to reduce	18 min.	41 min.	33 min.	28 min.	Not reduced
Bacteria No.	14,000,000	4,100,000	3,600,000	8,000,000	0
Complete Reduc.	Plus	Plus	Plus	Plus	Minus

Experiment III Part 6

Bacillus Prodigiosus

Sample No.	1	2	3	4	5
Time to reduce	20 min.	24 min.	40 min.	1hr.10min.	Not reduced
Bacteria No.	25,000,000	35,000,000	15,000,000	12,500,000	0
Comp.Reduc.	Plus	Plus	Plus	Plus	

Sample No.	1	2	3	4	5
Time to reduce	30min,	35min.	26min.	42min.	Not reduced
Bacteria No.	24,000,000	22,000,000	28,000,000	20,000,000	0
Comp. Reduc.	Plus	Plus	Plus	Plus	

Experiment III Part 7

Bacillus Mycoides

Sample No.	1	2	3	4	5
Time to reduce	50"	54"	36"	42"	Not reduced
Bacteria No.	4,500,000	3,800,000	6,800,000	5,400,000	0
Complete Reduc.	Plus	Plus	Plus	Plus	Minus

Sample No.	1	2	3	4	5
Time to reduce	47"	40"	23"	37"	Not reduced
Bacteria No.	4,300,000	6,000,000	9,000,000	5,800,000	0
Complete reduc.	Plus	Plus	Plus	Plus	

Experiment III Part 8 -Micrococcus

Sample No.	1	2	3	4	5
Time to reduce	1hr.20min.	1hr.32min.	1hr.11min.	1hr.19min.	Not reduced
Bacteria No.	7,000,000	6,200,000	8,500,000	5,300,000	0
Complete Reduc.	Plus	Minus	Plus	Minus	

Sample No.	1	2	3	4	5
Time to reduce	55"	48"	1hr.5min.	50"	Not reduced
Bacteria No.	12,000,000	16,000,000	9,000,000	14,500,000	0
Complete reduc.	Plus	Plus	Minus	Plus	

Experiment III Part 9

Streptococcus

Sample No.	1	2	3	4	check 5
Time to reduce	1hr.10min.	55"	1hr.18"	1hr.12"	Not reduced
No. Bacteria	11,000,000	1,300,000	950,000	860,000	0
Complete Reduc.	Plus	Plus	Plus	Plus	

CHECK

Sample No.	1	2	3	4	5
Time to reduce	2hrs.3"	1hr.35"	58"	1hr.16"	Not reduced
No. Bacteria	230,000	830,000	1,250,000	960,000	0
Complete Reduc.	230,000	830,000	Plus	Plus	

Experiment III Part 10

Staph-pyogenes (aureus)

Sample No.	1	2	3	4	5
Time to reduce	1hr.5"	48"	1hr.30"	1hr.17"	Not reduced
No. Bacteria	830,000	1,200,000	900,000	820,000	0
Complete Reduc.	Plus	Plus	Plus	Plus	

Sample No.	1	2	3	4	5
Time to reduce	2hr.5"	1hr.10"	1hr.35"	55"	Not reduced
No. Bacteria	300,000	920,000	8,500,000	1,100,000	0
Complete Reduc.	Plus	Plus	Plus	Plus	

Experiment III Part 11

Bacillus Vulgatus

Sample No.	1	2	3	4	5
Time to reduce	1hr.45"	1hr.16"	58"	2hrs.30"	Not reduced
No.Bacteria	1,100,000	1,500,000	200,000	510,000	0
Complete Reduc.	Plus	Plus	Minus	Minus	

Sample No.	1	2	3	4	5
Time to reduce	52"	1hr.12"	56"	1hr.18"	Not reduced
No.Bacteria	22,000,000	1,300,000	1,900,000	1,600,000	0
Complete Reduc.	Plus	Plus	Plus	Plus	

Experiment III Part 12

Bacillus Butyricus

Sample No:	1	2	3	4	5
Time to reduce	1hr.32"	1hr.5"	2hrs.30"	1hr.18"	Not reduced
Bacteria No.	960,000	1,600,000	325,000	1,400,000	0
Complete Reduc.	Plus	Plus	Plus	Plus	

Sample No.	1	2	3	4	5
Time to reduce	2hrs.18"	1hr.53"	55"	1hr.59"	Not reduced
Bacteria No.	410,000	540,000	1,500,000	300,000	0
Complete Reduc.	Plus	Plus	Plus	Plus	Plus

Experiment III Part 13

Bacillus Typhosus

Sample No.	1	2	3	4	5
Time to reduce	40"	56"	1hr.5"	1hr.30"	0
No. Bacteria	2,100,000	1,520,000	1,200,000	1,200,000	--
Complete or Incomplete Reduc.	Plus	Plus	Plus	Plus	Minus

Sample No.	1	2	3	4	5
Time to reduce	43"	1hr.12"	50"	2hr.10"	0
No. Bacteria	1,860,000	1,100,000	1,300,000	500,000	--
Complete of Incomplete Reduc.	Plus	Plus	Plus	Plus	Minus

Experiment III Part 14

Bacillus Pneumonia

Sample No.	1	2	3	4	5
Time to reduce	2hrs.30"	1hr.55"	2hrs.5"	1hr.10"	0
No. Bacteria	210,000	330,000	320,000	820,000	--
Complete or Incomplete Reduc.	Plus	Plus	Plus	Plus	Minus

Sample No.	1	2	3	4	5
Time to reduce	3 hrs.	2hrs.45"	2hrs.20"	3hrs.25"	
No. Bacteria	350,000	175,000	220,000	150,000	
Complete or Incomplete Reduc.	Plus	Plus	Plus	Plus	

It is evident from the previous experiments that pure cultures vary considerably in their reduction power. All of the organisms have the power of reducing, but with some the reduction was more complete than with others. B. Subtilis, B. Coli and Yeast seemed to have the greatest reduction power. Bacillus Acidi lactici gave rather an incomplete reduction, compared with the other organisms. It was noticeable also in Experiment II that the acid-producing organisms did not have as great a reducing powef as other organisms. Hence, the results in this experiment prove quite conclusively that the acid producing organisms do not have as great a reduction power as the other organisms.

Experiment IV

In reviewing the work of previous investigators, it was found that each investigator used a different strength of the Methylene blue solution. In some cases formalin was added to the Methylene solution, but the results of their experiments proved quite conclusively that the methylene-formalin solution was not a practical indicator for this test. Consequently this experiment was conducted with different strengths of methylene blue in order to find whether or not the various strengths of Methylene blue influence the time required for reduction.

As stated in the beginning of this thesis, two tenths (.2) of a cubic centimeter of methylene blue was added to each tube of milk in all previous experiments. In this experiment various dilutions and various strengths of methylene blue were used. Each of these will be described.

For each part the same milk was used so that the bacterial count was the same in each table.

Experiment IV Part 1

In this part of the experiment the same strength of methylene blue, as that used in the previous experiment, was diluted 1 to 50, 1 to 100, 1 to 150, 1 to 200, and 1 to 500. The results were as follows:

Sample No.	Dilution	Amts. added	Time required	No. Bacteria
1	1-50	.2c.c	1 min.	1,870,000
2	1-100	.2 c.c.	1 min.	"
3	1-150	.2 c.c.	At once	"
4	1-200	.2 c.c.	At once	"
5	1-500	.2 c.c.	At once	"

Sample No.	Dilution	Amt. added	Time required	No. Bacteria
1	1-50	.2 c.c.	1hr.5min.	960,000
2	1-100	.2 c.c.	49min.	"
3	1-150	.2 c.c.	41 min.	"
4	1-200	.2 c.c.	28 min.	"
5	1-500	.2 c.c.	7 min.	"

Experiment IV Part 2

In this part of the experiment the methylene blue was not diluted but different amounts of the methylene blue were added to the tubes of milk as follows:

.1 c.c.; .2 c.c.; .3 c.c.; .4 c.c.; and .5 c.c.

The same milk was used in each table thruout this part of the experiment so that the bacterial count was the same in each sample. The results were as follows:

Sample No.	Amt. Added	Time Required	No. of Bacteria
1	.1 c.c.	7 min.	25,000,000
2	.2 c.c.	12 min.	25,000,000
3	.3 c.c.	17 min.	25,000,000
4	.4 c.c.	21 min.	25,000,000
5	.5 c.c.	25 min.	25,000,000

Sample No.	Amt. Added	Time Required	No. of Bacteria
1	.1 c.c.	31 min.	5,400,000
2	.2 c.c.	39 min.	5,400,000
3	.3 c.c.	48 min.	5,400,000
4	.4 c.c.	1 hr. 5 min.	5,400,000
5	.5 c.c.	1 hr.19 min.	5,400,000

Sample No.	Amt. Added	Time Required	No. of Bacteria
1	.1 c.c.	40 min.	3,700,000
2	.2 c.c.	53 min.	3,700,000
3	.3 c.c.	52 min.	3,700,000
4	.4 c.c.	61 min.	3,700,000
5	.5 c.c.	1hr. 6 min.	3,700,000

Sample No.	Amt. Added	Time Required	No. of Bacteria
1	.1 c.c.	2 hrs. 15 min.	350,000
2	.2 c.c.	2 hrs. 34 min.	350,000
3	.3 c.c.	2 hrs. 39 min.	350,000
4	.4 c.c.	3 hrs. 7 min.	350,000
5	.5 c.c.	3 hrs. 11 min.	350,000

Sample No.	Amt. Used	Time Required	No. of Bacteria
1	.1 c.c.	29 min.	6,600,000
2	.2 c.c.	31 min.	6,600,000
3	.3 c.c.	43 min.	6,600,000
4	.4 c.c.	51 min.	6,600,000
5	.5 c.c.	46 min.	6,600,000

The results of the different parts of this experiment indicate that different strengths and different dilutions of the methylene blue solution cause variation in the time required for reduction. With the different dilutions, when the bacterial content was high, the reduction took place at once. If there are only a few bacteria present in the milk the dilution method might be used; otherwise a stronger solution of methylene blue should be used. The test was much more successful when different amounts of the methylene blue solutions were added to the milk without dilution. The results show that the greater the amount of methylene blue added to the milk the longer the time required for reduction. Thus proving that it would be necessary to have a different reduction table for each strength of the methylene blue solution.

Experiment V

All of the expetiments thus far were conducted at a temperature of 40 to 45 degrees C. This experiment was conducted at the following temperatures: 18 degrees C.; 31 degrees, C; 37 degrees C.; 43 degrees C.; 50 degrees C.; 60 degrees C.; and 70 degrees C.; in order to determine the effect of different temperatures on the time required for reduction. The milk used was from the same vessel in each part of the experiment. Tow tenths of a cubic centimeter of methylene blue was used in each tube. The results were as follows:

Experiment V Part 1

Sample No.	Temperature	Time required	Bacteria Number
1	18 degrees C.	50 min.	13,800,000
2	31 " C.	38 min.	13,800,000
3	37 " C.	26 min.	13,800,000
4	42 " C.	24 min.	13,800,000
5	50 " C.	23 min.	13,800,000
6	60 " C.	35 min.	13,800,000
7	70 " C.	1hr. 5 min.	13,800,000
8	85 " C.	Did not reduce	"

Experiment V Part 2

Sample No.	Temperature	Time Required	Bacterial Number
1	18 degrees C	1 hr. 17 min.	1,320,000
2	31 " "	1 hr. 10 min.	"
3	37 " "	59 min.	"
4	40 " "	57 min.	"
5	45 " "	57 min.	"
6	50 " "	56 min.	"
7	60 " "	48 min.	"
8	70 " "	1 hr. 58 min.	"
9	85 " "	Did not reduce	"

Sample No.	Temperature	Time Required	Bacterial Number
1	18 degrees C	1 hr. 10 min.	19,100,000
2	33 " "	58 min.	"
3	35 " "	39 min.	"
4	37 " "	20 min.	"
5	45 " "	15 min.	"
6	50 " "	16 min.	"
7	60 " "	23 min.	"
8	70 " "	----	"
9	80 " "	----	"

Sample No.	Temperature	Time Required	Bacterial Number
1	18 degrees C	2 hrs. 55 min.	8,700,000
2	33 " "	1 hr. 42 min.	"
3	35 " "	1 hr. 3 min.	"
4	37 " "	55 min.	"
5	45 " "	33 min.	"
6	50 " "	34 min.	"
7	60 " "	----	"
8	70 " "	----	"
9	80 " "	----	"

From the results of the previous experiment it was decided that the time required for the reduction decreased as the temperature increased until the temperature reached a point where a part of the bacteria were killed, or became inactive. These results also showed that it would be necessary to have a different reduction table for each variation in temperature of ten degrees Centigrade. The temperature of 40 to 45 degrees C., no doubt, gave the best results.

Experiment VI

In reviewing the work of various investigators it was found that they had used different kinds of stoppers for plugging the tubes after methylene blue had been added. Some of the investigators were of the opinion that the reduction process was hastened a certain per cent if the oxygen was excluded. Consequently this experiment was conducted in order to determine the influence of the presence of air. Each of the five samples of milk, constituting one of the following

table

tables, were identical. Various methods were used in excluding the air. Results were as follows:

Experiment VI Part 1

Sample No.	Kind of Stopper Plugs	Time of Reduction	No. Bacteria
1	With paraffin	2hrs. 17 min.	860,000
2	" Oil	2 hrs. 5 min.	"
3	" Cotton	2 hrs. 23 min.	"
4	" Rubber	2 hrs. 20 min.	"
5	With-out Stopper	2 hrs. 28 min.	"

Sample No.	Kind of Stopper	Time Required	Number Bacteria
1	Paraffin	50 min.	4,700,000
2	Oil	55 min.	"
3	Rubber	59	"
4	Cotton	59 min.	"
5	Without stopper	58 min.	"

Sample No.	Kind of Stopper	Time of reduction	No. Bacteria
1	Paraffin	1 hr. 15 min.	1,150,000
2	Oil	1 hr. 22 min.	"
3	Cotton	1 hr. 14 min.	"
4	Rubber	1 hr. 25 min.	"
5	Without stopper	1 hr. 19 min.	"

From the results of this experiment it is evident that the manner in which the tube was stoppered did not have any effect on the time required for reduction. The tubes that were not plugged at all reduced in practically the same length

of time as those which were stoppered with melted paraffin. It probably would be advisable, however, to use cotton for stoppering the tubes, in order to keep out foreign material.

Experiment VII

The work in this experiment was conducted in order to determine the effect of an antiseptic upon the reduction process. Some of the work was conducted with milk which had been drawn under aseptic conditions in order to determine the effect of secretions upon the reduction test. After chloroform was added to the milk it was allowed to stand for a period of thirty minutes before the methylene blue was added. The results were as follows:

Experiment VII Part 1

Sample No.	Amt. Chloroform Added	No. Bacteria 30 min. after Chloroform was Added	No. Bacteria	Time Required
1	None		580,000	3hrs.25"
1	.3 c.c.	None		Not reduced
2	None		630,000	3 hrs. 15"
2	.3 c.c.	None		Not reduced

Sample No.	Amt. Chloroform Added	No. Bacteria 30 min. after Chloroform Was Added	No. Bacteria	Time Required
1	None		1,800,000	1 hr. 30"
1	.3 c.c.	16000		Not reduced
2	None		2,200,000	1 hr. 5"
2	.3 c.c.	18000		Not reduced

Sample No.	Amt. Chloroform Added	No. Bacteria 30 min. after Chloroform was added	No. Bacteria	Time Required
1	None		2,800,000	1 hr. 18"
1	.3 c.c.	600		Not reduced
2	None		1,670,000	2 hrs. 12"
2	.3 c.c.	1100		Not reduced

Sample No.	Amt. Chloroform Added	No. Bacteria 30 min. after chloroform was added	No. Bacteria	Time Required
1	None		480,000	3 hrs. 18"
1	.5 c.c.	None		Not reduced
2	None		670,000	2 hrs. 25"
2	.5 c.c.	None		Not reduced

Sample No.	Amt. Chloroform Added	No. Bacteria 30 min. after chloroform was added	No. Bacteria	Time Required
1	None		930,000	1 hr. 55"
1	.5 c.c.	None		Not reduced
2	None		540,000	2 hr. 10"
2	.5 c.c.	None		Not reduced

Sample No.	Amt. Chloroform added	No. Bacteria 30 min. after chloroform was added	No. Bacteria	Time Required
1	None		860,000	2 hrs. 15"
1	.5 c.c.	None		Not reduced
2	None		1,250,000	1 hr. 37"
2	.5 c.c.	None		Not reduced

Experiment VII Part 2

This part of the experiment was performed with milk which was drawn under aseptic conditions and was conducted in order to determine the reduction power of freshly drawn milk, also to determine whether or not reduction was caused by secretion. The temperature used in the experiment was 37 degrees C. The results were as follows:

Cow No.	Kind of Milk	Time Required	Number Bacteria	No. Bacteria after 24 hrs.
1	Fore Milk	18 hrs.	1260	18,000
1	Middle Milk	Not reduced 24 hrs.	660	9,100
1	After Milk	Not reduced 24 hrs.	420	11,000

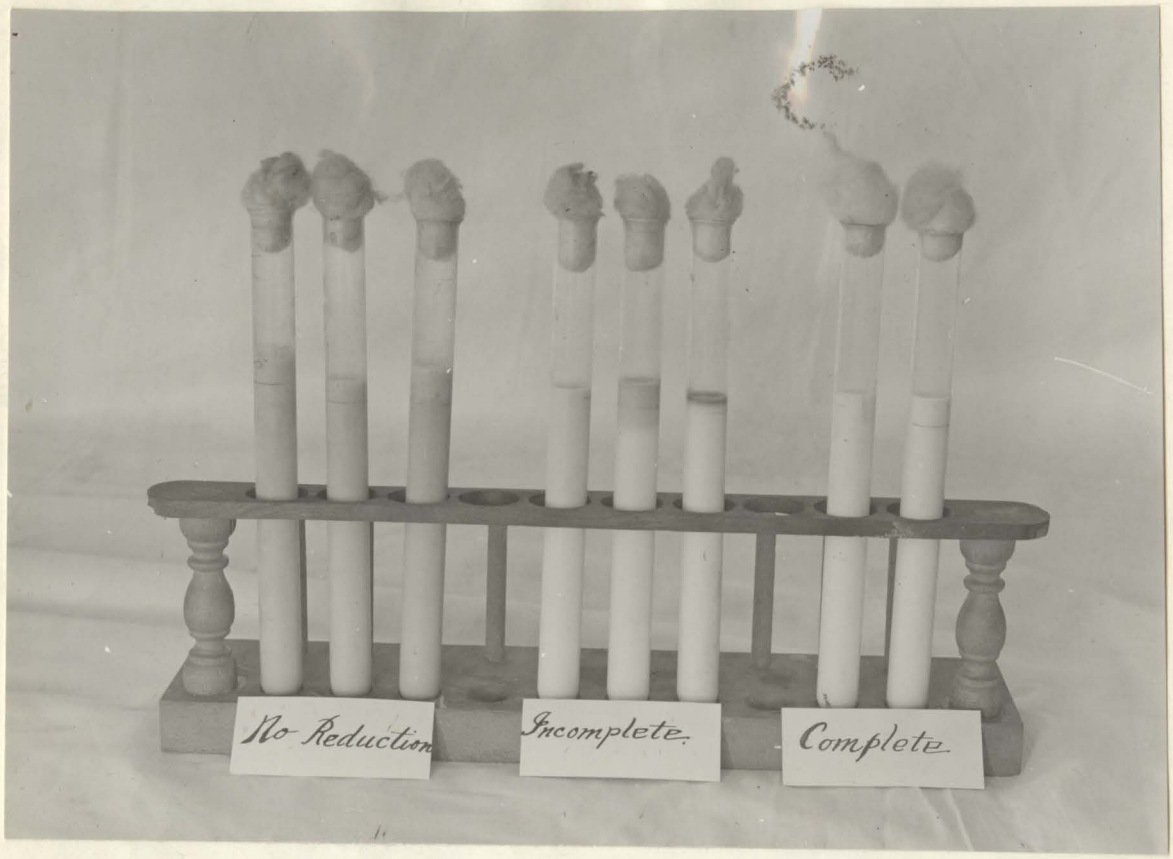
Cow No.	Kind of Milk	Time Required	No. Bacteria	No. Bacteria after 24 hrs.
2	Fore Milk	12 hrs.	2900	27,000
2	Middle "	Not reduced 24hrs.	810	15,000
2	After "	" " "	470	17,200

Cow No.	Kind of Milk	Time Required	No. Bacteria	No. Bacteria after 24 hrs.
3	Fore Milk	16 hrs.	4800	32,000
3	Middle "	Not reduced	900	21,000
3	After "	" "	950	22,000

Cow No.	Kind of Milk	Time Required	No. Bacteria	No. Bacteria after 24 hrs.
1	Middle	1 hr. 55 min.	786,000	Three days old
2	"	1 hr. 37 min.	980,000	" " "
3	"	2 hrs. 35 min.	550,000	" " "

Reduction did not take place in any of the samples of milk which contained chloroform, presumably because of the lack of living bacteria. The samples of the same milk which did not contain chloroform and which contained large numbers of bacteria

reduced the methylene blue in a reasonable length of time. From these results it appears that reduction does not take place under conditions which are not favorable to the growth of bacteria. In the samples of milk which were taken under aseptic conditions reduction did not take place until the bacteria had time to develop, thus proving that the reduction is caused by an enzyme secreted by bacteria during their growth.



Tubes of milk reduced and not reduced.

Summary

1. The time required for the reduction of methylene is practically the same for any given number of bacteria.

2. The time required for reduction cannot be estimated exactly on account of slight variations.

3. All bacteria commonly found in milk have the power of reduction.

4. B. Coli, B. Subtilis, and yeast prove to have the greatest reduction power.

5. Acid-producing bacteria require a longer time for reduction than other bacteria.

6. The percent of acidity does not affect the time required for reduction.

7. Different strengths of methylene blue require different lengths of time for reduction.

8. Temperature modifies the time of reduction.

9. Reduction temperatures vary from 18 degrees C to 60 degrees C, with the greatest activity at from 40 degrees to 45 degrees C.

10. The presence or absence of oxygen has noevident effect on the time of reduction.

11. The reduction is checked by the use of an antiseptic.

12. The reduction probably is caused by an enzyme secreted by bacteria during their growth in milk.

13. The methylene blue reduction test is an efficient test for determining approximately the number of bacteria in milk.

BIBLIOGRAPHY

1. Duclaux, Le Lait, etudes chimiques et microbiologiques. Paris 1887, S. 1.
2. Munchener mediz. Wochenschr. 1900, No. 37.
3. Analyst 1901, 26, 148.
4. Diese Zeitschrift 1902, 5, 1113.
5. Archives fur Hygiene 1902, 44, 295.
6. Milch-Ztg 1903, 32, 625 und Revue Generale du Lait 1904, 1, 505.
7. Hygienische Rundschau 1904, 14, 1137.
8. Archives fur Hygiene 1906, 58, 313.
9. Diese Zeitschrift 1904, 7, 81.
10. Milchwirtschaftliches Zentralblatt 1907, 3, 41.
11. Zeitschrift fur Hygiene und Infektionkrankheiten 1906,52,161.
12. Orla Jensen, Oversigt over det Kgl. Danske Videnskabernes Selskabs Forhandlinger, 1906. No. 5.
13. Zeitschrift fur Hygiene und Infektionskrankheiten 1907, 58, 1.
14. Milchwirtschaftel Zentralbatt 1908, 4, 49.
15. Zeitschrift fur Untersuchung. der Nahrungs - und Genussmittel 15, Jan-June 1908, pp 385.
16. Deise Zeitschrift 1906, 11, 377.
17. Milchwirtsch Zentbl. 41, No. 6, 168-172.
18. Milchwirtsch Zentbl., 4, 156-175.
19. Centrbl Bakt. Abt. Orig., 49, 1909.
20. Milchw. Zentbl., 41, 1912, No. 2, pp 40-44, No. 3 pp 68-74, No. 4 pp 104-105, No. 5, pp 134-140.
21. Centrbl. Bakt. Abt. Orig., 1912, 17, pp 391.
22. Moores Laboratory Manual 3rd edition pp 36.
23. Bacteria in Milk and Its Products. Conn. pp 269.