AN INVESTIGATION AS TO THE INCONSISTENCIES OF THE METHYLENE BLUE REDUCTION TEST AND MEANS OF CONTROLLING SAME

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By

Horace Edward Alphin
Blacksburg, Virginia

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Approved:

H.D. Massey

J.L. Wilson

H.W. Rees

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INTRODUCTION

Much investigation in late years has been given to the improvement of methods for testing the material quality of milk. The growth of bacteria in milk usually involves a fermentation of the milk sugar followed by splitting of the proteins. Because of the rapid increase of bacteria in milk, the bacterial count has been widely accepted as a criterion of the keeping quality; or of its past history and fitness for human consumption. In an effort to meet such demands several testing methods have been developed, but most of them are tedious and require skilled technicians to carry them out, involving much time and equipment. However, each method is a measure of a separate and distinct phenomenon and the improvement of one method will not act to supplant, but will probably supplement, another.

In 1900 it was suggested that the reduction of methylene blue when added to milk may be used as an indication of its bacterial content. The intervening thirty-five years have witnessed the adaptation of this

suggestion, and today the test is being more generally used. This dye has not been used because of the precise knowledge of its behavior in milk, but its value has been largely indicated by experience plus trial and error methods.

The methylene blue reduction test has the advantage of being simple to operate, easy to interpret results, and requires little expense for materials. The test is based on the principle that the color imparted to milk disappears more or less quickly depending on whether the bacterial content is high or low. Despite all of the advantages of the test, there are a number of factors which directly influence the consistency of results. A survey of literature on this subject reveals the fact that several kinds of variability have been studied. The question arising, after reading all available literature on the test, is: "What effect does dissolved oxygen have on reduction time and does the dissolved oxygen content of milk vary when milk is produced under average conditions?"

It seems reasonable to expect that a milk high in bacterial numbers and high in dissolved oxygen may retain its blue color for several hours, thus indicating a milk with a low bacterial content. While a milk with a low bacteria count and a low dissolved oxygen

content may reduce in a shorter time, indicating a milk with a high bacterial content. Such irregularities are not uncommon. The object of this study is to determine the cause of such fluctuations in the test and develop a technique to correct them.

REVIEW OF LITERATURE

After making a study of the literature on the methylene blue reduction test it is interesting to note that considerable work has been devoted to certain phases of variation, while other factors causing variation in the test have been practically free from investigation.

Thornton and Hastings (1) brought out the fact that there i s no oxygen in the methylene blue molecule and reduction of the dye involves a transfer of hydrogen. According to their studies, two molecules of hydrogen are involved. One molecule of hydrogen draws the chlorine molecule from the basic terminal nitrogen. A shifting of the double bond takes place leaving the bridged nitrogen unsatisfied.

Barthel (2) believes that the disappearance of methylene blue in milk takes place in two stages:

1. The removal of dissolved oxygen by the growing bacteria.

2. The reduction of dye by constituents of the milk.

Fred and Chappelear (3) made the important observation that Steptococcus lactis is a greater consumer of oxygen than other organisms commonly found in milk. They also counted the bacteria at hourly intervals until reduction took place. Their data gave the relation between reduction and the number of cells and reveals that Barterium denitrificans is a rapidly growing organism, but a slower reducer of methylene blue when added to milk.

Before the oxygen can be reduced it is indispensible that the molecular oxygen be consumed or combined in some way. According to the theory of Wieland (4) the molecular oxygen dissolved in milk may be reduced by hydrogen first forming hydrogen peroxide, which is then reduced to water.

Fay (5) found that the oxidation reduction potential of milk becomes more negative as the molecular oxygen disappears. At the more positive values the oxygen has a greater affinity for the hydrogen than the methylene blue. He also reports that the potential zone of reduction of methylene blue in skim milk is more negative than that observed for cream.

No reports have been found in the literature, on the study of the gas content of milk, since that of

Marshall (6) in 1902. We gather from his studies that as milk leaves the udder there is a diminution in the amount of carbon dioxide and an increase in the amount of oxygen to a certain percentage which is dependent on the thoroughness of aeration.

Thornton and Hastings (7) are of the opinion that the variation in the oxygen content of milk produced in the ordinary way does not introduce any inaccuracies; however, their conclusions were drawn from the percentages of oxygen which Marshall (6) obtained in his analyses.

The work of Robertson and Frayer (8) shows that there is a variation in the plate count on milks of low bacterial content and there is less variation in the methylene blue reduction test on milks of high bacterial content.

Gebhard (9) has shown the bleaching effect of light to be more intense in the absence of oxygen provided that the available light consists of waves shorter than 620 M.

Aikins and Fay (10) found that the oxidation reduction potential of milk samples exposed to sunlight becomes more negative immediately after exposure. This negative value continues until approximately an Eh value of zero has been reached. When comparing the reduction time of two samples of milk containing methylene blue,

they found that the visible reduction of the samples exposed to light preceded the one in the dark by 2.5 hours.

Hall (11) brings out the fact that a small amount of alkali when added to milk containing methylene will accelerate the change to its leucobase. The milk containing the least alkali is always last to lose its color when the number of bacteria per cc. is consistent.

Johns (12) endeavors to show the value of preliminary incubation at a temperature of 55 degrees Fahrenheit, which should indicate the probable degree of keeping quality with a greater degree of accuracy than most other tests. This modified test encourages the multiplication of bacteria capable of growth at a temperature of 55 degrees F. and also at blood temperature.

Barthel (2) has reported the inaccuracies of the test to be as follows:

1. The rate of oxygen consumed by the bacteria varies with the different species.

2. The removal of bacteria from the body of the milk by rising butterfat.

Hastings (1) states, "that any variation of pH. met with in the test is not sufficient to have any measurable visual effect on the reduction time." He found that the number of bacteria must reach 100 million before any appreciable change is noted in the reaction of the milk

as measured by titrations.

Davenport, Hastings and Wright (13) observed that a 1-20,000 solution of methylene blue should impart deep enough color to the milk so that it may be observed readily. It was noted that the reduction time was prolonged as the concentration of the dye was increased.

Thornton and Hastings (7) believe that the leucocytes are important in consuming oxygen and are a factor in the reduction of methylene blue in milk. Their assumption was made on basis of the fact that bacteriologists frequently use plant and animal tissue to produce anaerobic conditions.

It is suggested by Whitehead (14) that sunlight catalyses an oxidation reduction reaction in which unsaturated fats are oxidized and the methylene blue is reduced, thus he has emphasized the necessity of performing the test in the dark.

Dubas (15) has demonstrated that sterile milk when protected from the atmosphere by a vaseline seal is capable of reducing a number of dyes, including methylene blue.

Chappelear and Fred (3) made a comprehensive study of eight outstanding dyes, and found that they reduce over a potential range negative to that of methylene blue. The sensitiveness toward the dyes appears to

increase with the fall in potential.

PRESENT INVESTIGATION

Purpose of Study-- A review of the literature, and experience in operating the test, has emphasized the need of a study of the factors influencing the test and how these may be controlled in order to eliminate the inaccuracies thereby making the test more dependable, hence more widely applicable.

Each factor which probably influences the variation in the reduction of methylene blue will be dealt with separately. These studies have been developed along the following lines:

1. The effect of dissolved oxygen on reduction time.
2. The effect of methods of mixing and sampling milk and adding dye on reduction time.
3. Controlling reduction time by the addition of salts and other substances.
4. The effect of methylene blue on the growth curve of bacteria which frequent market milk.

THE EFFECT OF DISSOLVED OXYGEN ON REDUCTION TIME

Efforts were made to find the real relationship between the reduction of methylene blue and the dissolved oxygen content of milk. This problem was approached by making several preliminary tests to determine the influence of the oxygen content on reduction time.

Treating Milk Samples with Carbon Dioxide-- Attempts have been made to displace the oxygen in milk with carbon dioxide, in this way hoping to show that low dissolved oxygen hastens reduction.

The method which was employed consisted in washing the milk or displacing the oxygen of the milk with carbon dioxide. Since carbon dioxide is heavier than oxygen it could be forced into a tube of milk to drive out the oxygen. The procedure consisted in dividing a sample of market milk into two parts: to one part was added the standard amount of methylene blue commonly employed in the reductase test. Into the second part carbon dioxide was bubbled. This was done by repeatedly exhausting the atmospheric air from the flask and then admitting carbon dioxide, so as to be certain that the

greater portion of the oxygen had been expelled. The later portions of the oxygen were given off reluctantly. This sample was closed up tightly to exclude the entrance of atmospheric gases. Both samples were then incubated at a temperature of 37 degrees Centigrade until the methylene blue was reduced to its leucobase. Since a milk of relatively low bacterial count was used, the sample which received no treatment reduced in five hours and ten minutes, while the sample into which carbon dioxide was bubbled reduced in three hours and ten minutes. From this it would seem that the dissolved oxygen content causes a fluctuation in the reduction time of methylene blue when added to milk. In view of this fact it became necessary to determine whether the aeration of milk would bring about any change in the dissolved oxygen content, or influence the reduction of methylene blue when added to a sample of milk.

The Dissolved Oxygen Content of Milk

A brief review of literature seems to indicate that there is no accurate method avialable for determining the oxygen content of milk. The technique which Marshall (6) used may rightly be condemned and he states, "it was not his purpose to ascertain the amount of gases

that can be drawn from milk but rather to secure a quantitative examination of the changes which take place under aeration."

Preliminary Method-- Since no method to measure the amount of oxygen in milk could be found, it became mandi-tory for the author to devise a method. It was first thought that the Winklers (16) method for water analyses might be the most satisfactory, but because of the complex substances found in milk it was impossible to utilize the test even when the milk was diluted, with water of a known content. The results obtained proved conclusively that this method is very unsatisfactory for determining the dissolved oxygen content of milk.

Heat was next used to drive the gases from a definite quantity of milk. After a number of preliminary heatings it was found that the gases could be driven off by heating the milk to a temperature of 104 degrees Centigrade.

After the gas had been collected in a graduated tube it was essential that some quick yet accurate method for determining the amount of oxygen and carbon dioxide be employed. The Orset (17) gas method was first used, but owing to the fact that such a small amount of gas was available it became very difficult to make accurate

analyses. A slight modification of this test was tried, which enabled slightly better results to be secured, but the procedure for this method was too long and involved too much equipment for rapid determinations. A modification of Henderson's (18) Syringe Gas Analizer was finally resorted to for making the gas analyses. This method has less than one per cent error for oxygen and .5 per cent for carbon dioxide.

Description of Method-- The apparatus used in making the analyses is described in Figure 1, on the following page.

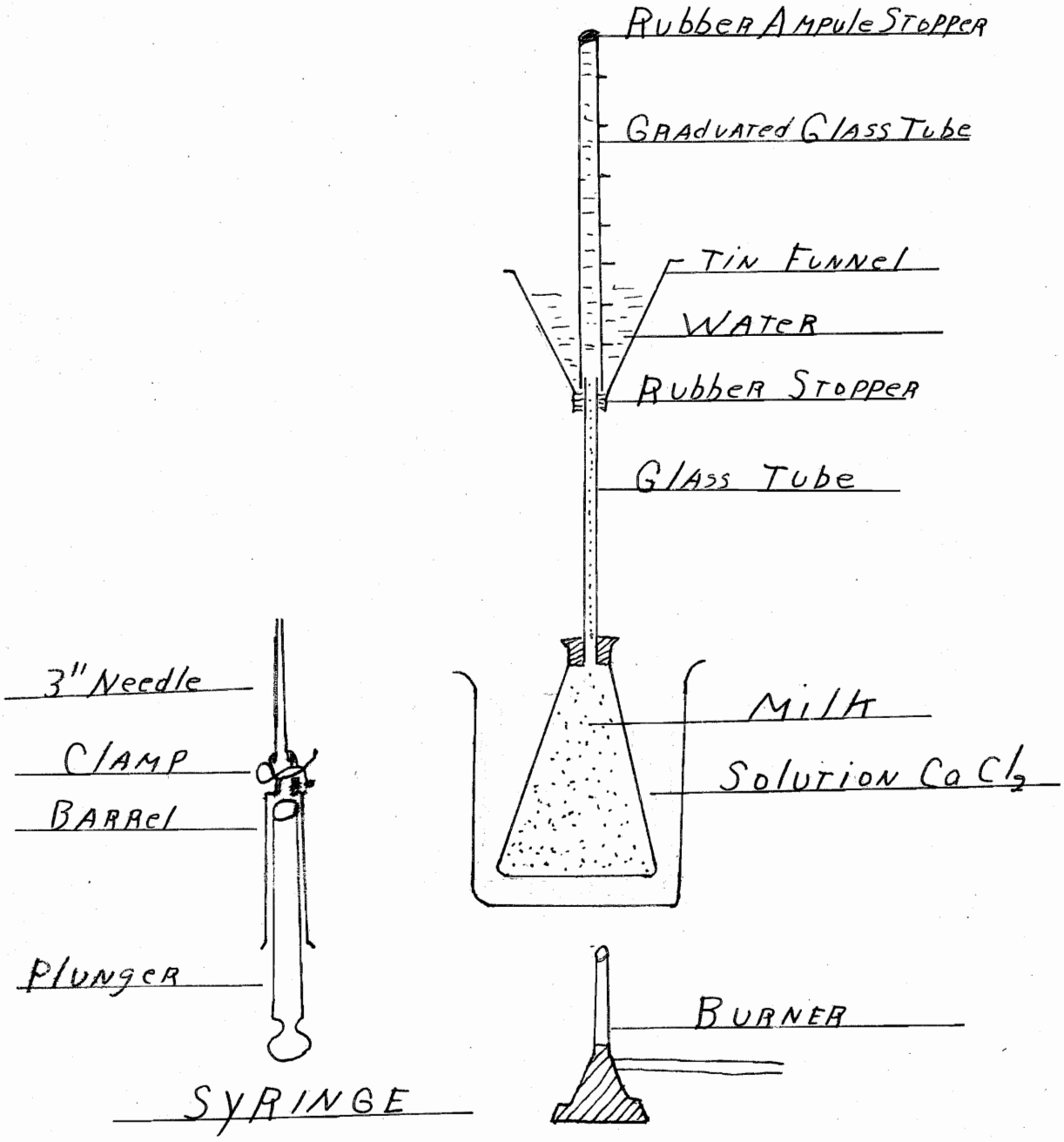


Figure 1. Apparatus used to determine the dissolved gases of milk.

The reagents used in making the analyses are as follows:

1. Carbon Dioxide Absorbent

This absorbent is made by dissolving 10 grams of potassium hydroxide in 100 cubic centimeters of water.

2. Oxygen Absorbent

A powder is prepared consisting of 100 grams of sodium sulphite mixed with 10 grams of anthraquinone-beta-sulphonic acid. The absorbent is a red liquid made by dissolving 2 grams of the powder in 10 cc. of a normal solution of potassium hydroxide. The reagent becomes colorless through the action of absorbed oxygen. It was preserved by being covered with mineral oil.

Procedure-- The procedure consists in filling a small Erhlermyer flask level full with milk and inserting a rubber stopper, containing a short glass tube, into the mouth of the flask. The displaced milk is forced up the tube completely filling it. Another rubber stopper is fitted into the bottom of a tin funnel and placed over the other end of the small glass tube. The funnel is then filled with water above the glass tube now holding the milk. A short graduated glass tube containing a rubber ampide stopper on one end is filled with water, and the

other end is placed over the small glass tube which is in the bottom of the funnel under water. The flask containing the milk is placed in a basket containing sufficient calcium chloride to raise the boiling point to a temperature of 104 degrees Centigrade. This temperature has to be obtained in order for the gases to be released. When heat is applied the gases are driven off and displace the water in the graduated glass tube.

After the gas has been collected, it is essential that an accurate method of analysis be employed. It was found that a modification of the Henderson's Syringe gas Analyzer would be best suited for this purpose. The syringe which was used in making these analyses is described in Figure 1.

In drawing a sample of gas the needle is thrust through the rubber seal and the plunger of the syringe is worked back and forth two or three times while the pinchcock is still open. After drawing off the gas plus two or three cc. of water the pinchcock is allowed to close and syringe is withdrawn from the rubber seal. As soon as the syringe has lost the heat imparted by the hand, it is stood upright and the volume of gas is read. A magnifying glass aids somewhat in the precision of readings.

The needle of the syringe is now dipped in

the carbon dioxide absorbent, and two or three cc. are drawn into the syringe. The syringe is shaken until no further absorption of gases takes place, and is then stood upright with its weight resting on the plunger. As soon as the gas has reached room temperature, the second reading is made. When two successive readings are identical, they are taken as indicating the correct volume. When the initial volume is divided into the difference between the two readings, the result represents the percentage of carbon dioxide in total gas.

In determining the percentage of oxygen two or three cc. of the oxygen absorbent is drawn into the syringe and the same procedure that was used in making the carbon dioxide analyses is followed. This absorbent has the advantage of being thin, with a red color which fades out when its absorbent powers have been exhausted. The per cent of oxygen in the total gas is obtained by dividing the initial volume into the difference between the two readings.

All gas remaining in the syringe (nitrogen, hydrogen and hydrogen sulphide) will be recorded as residual gas.

The milk used in each group of analyses was produced under three conditions: Unexposed milk, thoroughly aerated milk, and milk which was produced in the ordinary way.

Analyses of Unexposed Milk

In order to find the changes which take place after milk leaves the udder, it becomes necessary to find the amount of gases in unexposed milk. An effort was made to test milk drawn in this way and compare it with milk which has been exposed to the atmospheric gases. Several devices have been tried to collect milk without exposing it to the air, but they require very specialized equipment. Especially is this true of the method used by Marshall (6), although it appears to be the most effective method previously adapted. Such a method is too cumbersome and the slightly added accuracy would normally not suffice to justify its use.

Apparatus Used-- In an effort to draw unexposed milk from the cow the following method was employed. A thin, flexible piece of rubber tubing, 14 inches long, was stretched tightly over the cow's teat and connected to a glass tube leading to the bottom of a vacuum flask. The rubber tubing was rolled tightly before being placed on the cow's teat and then it was allowed to unroll while the cow was being milked. When the rubber tube was full of milk the pinchcock was allowed to open and the milk was drawn into the vacuum flask. The pinchcock was always kept tightly closed except when milk was drawn

into the flask. This procedure was repeated until an adequate amount of milk had been drawn for the analysis. After drawing, the sample was immediately analyzed before any exchange in gases was allowed to take place.

Figure 2 will serve to illustrate the type of apparatus used in drawing the milk.

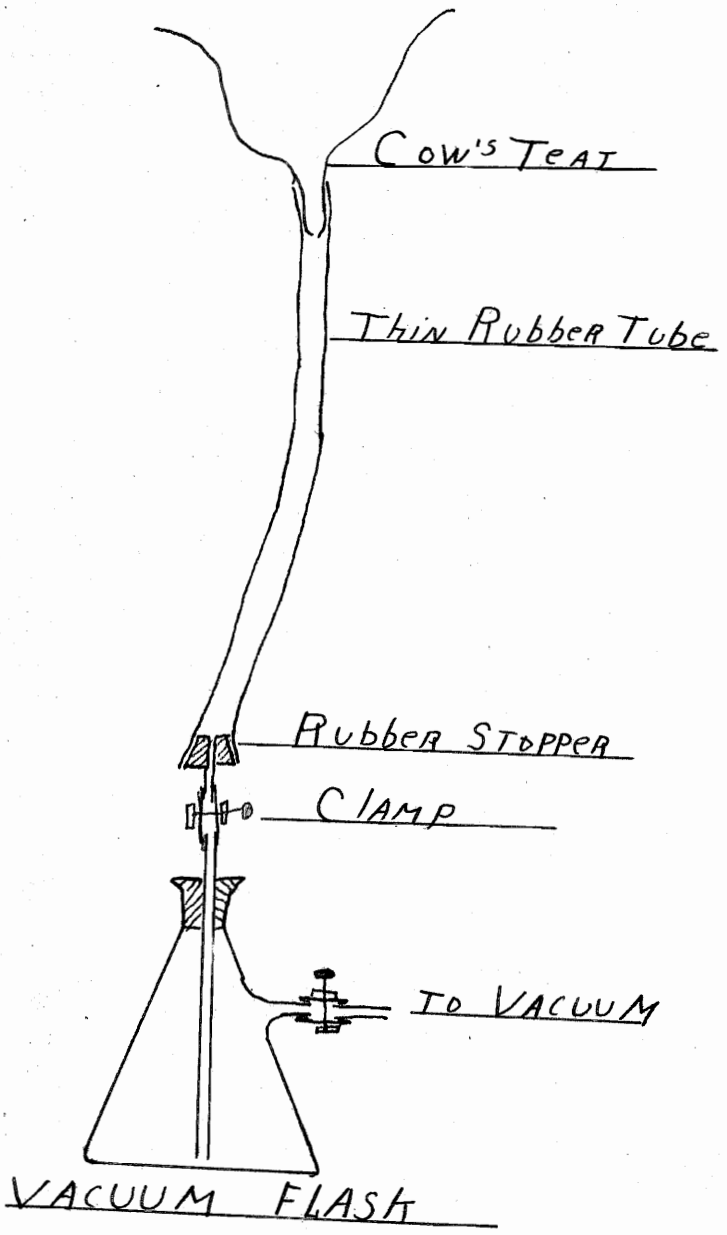


Figure 2. Apparatus used in drawing unexposed milk from the cow.

Table No. 1 Percent of gas in unexposed milk

Sample Number:	1	2	3	4
Vol. Milk Used	218	218	170	170
Total Gas in CC.	9.6	8.5	4.24	4.3
Free CO_2 in CC.	7.3	6.3	2.76	2.90
% CO_2	76.04	74.12	67.50	67.44
Free O_2 in CC.	.30	.30	.318	.40
% O_2	3.12	3.52	7.50	9.30
Res. Gas in CC.	3.0	1.80	1.86	1.00
% Res. Gas	20.84	22.36	25.00	23.26

The above four gas analyses represent the gas content of milk as it is drawn from the cow. The amount of carbon dioxide obtained in the analyses is given in percentage as follows: 76.04, 74.12, 67.50, and 67.44; with an average of 71.27 per cent for the four samples. Considering the extremes of the four analyses there appears to be no serious discrepancy. It is entirely possible and highly probable that the gas content varies, as is the case with the constituents of milk. Each volume of gas obtained in the analyses was corrected to a temperature of 24° C.

The percentage of oxygen for the first and last two samples is very uniform. It is surprising to find such low percentages of free oxygen in milk.

The percentage of oxygen falls far below that of air and also below the solubility of oxygen in water.

Oxygen was found in the following percentages: 3.12, 3.52, 7.50 and 9.30.

Since these figures are based on the percentage of gas volume obtained, they represent mere traces of oxygen per volume of milk. The average per cent of oxygen in the total gas for the four samples was 5.86. It is interesting to note that the two low percentages of oxygen were obtained when the cow was kept in the stable. The third and fourth samples were collected when the cow was allowed to run in the barn lot. There is not enough experimental evidence available at present to make any definite statement as to whether the oxygen percentage would be fluctuated by exercise received by the animal.

Analyses of Thoroughly Aerated Milk

After ascertaining the gas content of unexposed milk, the author planned to study the effect of excess aeration on the gases in milk and to determine if oxygen content of milk can be raised to a consistent percentage.

Treatment of Sample-- The milk used in this series of experiments was milked in the ordinary way and thoroughly aerated over glass. The aeration device was made by

inverting a large glass funnel into the top of another. A small stream of milk was poured over the top funnel so that the milk could be exposed to the largest possible surface. By exposing a film of milk over a large area it is thought that the maximum interchange of gases would take place. The aeration process was repeated several times to insure thorough aeration. The milk aerated over glass was analyzed in the same way as unexposed milk.

Results--

Table No. 2 Analyses of milk before and after aeration

Sample Number:	1	2	3	4	5	6
Vol. Milk Used	170	170	170	170	170	170
Total Gas in CC. before aeration	3.62	3.35	5.74	4.65	3.50	5.02
Total Gas in CC. after aeration	3.89	3.40	6.00	4.70	3.50	5.00
Free CO_2 in CC.	.69	1.00	2.80	1.50	.50	1.9
% CO_2 in Total Gas	22.00	29.54	46.66	31.91	14.28	38.00
Free O_2 in CC. before aeration	.43	.41	.57	.65	.47	.61
Free O_2 in CC. after aeration	.77	.77	1.00	1.00	1.00	1.00
% O_2 in Total Gas before aeration	12.00	12.50	10.00	14.00	13.50	12.20
% O_2 in Total Gas after aeration	18.00	22.72	16.66	21.27	28.57	20.00
Res. Gas in CC.	2.43	1.63	2.20	2.20	2.00	2.10
% Res. Gas.	60.00	47.74	36.68	46.81	57.15	42.00

Aeration of the above samples took place in a large laboratory at a temperature of 24 degrees Centigrade.

The gas samples were also analyzed at a constant temperature of 24 degrees Centigrade, and correction was made for any change in atmospheric pressure.

The percentages of oxygen in these tables are: 18.00, 22.12, 16.66, 21.27, 20.00 and 28.57.

The average of these six samples is 21.10 per cent.

This represents an increase of 15.24 per cent over the unexposed milk. It is noted that the percentages of oxygen in the gases of thoroughly aerated milk is about equal to that of the atmosphere. In these tables the percentages of carbon dioxide are: 22.00, 29.54, 46.66, 31.91, 14.28 and 38.00.

The average percentage for the six samples is 30.39.

There is a loss of 40.88 per cent of carbon dioxide when compared with unexposed samples of milk. After a certain minimum per cent of carbon dioxide has been reached there is apparently no further change due to excess aeration.

The residual gases in milk are thought to be mostly nitrogen, hydrogen and hydrogen sulphide, and the percentages for the six samples are as follows: 60.00, 47.74, 36.68, 46.81, 57.16 and 42.00.

We gather from these studies that as the milk leaves the udder of the cow there is an increase in the amount of oxygen and a decrease in carbon dioxide which is regulated by the thoroughness of aeration. This is brought about by the natural diffusion and solubility of gases. The exchange of gases is facilitated by a thin film of milk passing over a large surface. We do not find such a complete interchange of gases when the ordinary method of cooling is employed.

Analysis of Milk Produced in the Ordinary Way

One of the objectives of this investigation is to make analyses of the oxygen content of milk, when produced in the ordinary way, and to see if the results coincide with the figures obtained by Marshall (6). It seems reasonable to expect that a slight difference in the oxygen content of milk would cause a variation in the reduction time of methylene blue; especially when it is added to milk containing approximately the same number of bacteria per cubic centimeter. In view of this supposition the subsequent procedure was followed with milk which was produced by a number of farmers in the vicinity of Blacksburg, Va. When samples of milk were collected in this way they were thought to be representative of the

milk produced by farmers, under similar conditions.

Method of Study-- Each sample of milk was treated in the following manner: about twenty cc. of the sample was used in setting up the methylene blue reduction test in duplicate. Another portion of the sample was used in making the agar plate count. Usually a very small portion of the sample was used in making the microscopical count, and 170 cc. of the milk was used in making the gas analyses.

The amount of oxygen, carbon dioxide, and other gases were determined by the same method as outlined on pages 14 - 16. Care was always taken to use exactly the same procedure in testing each sample and correction was made for any change in temperature.

The methylene blue reduction test was set up by adding 10 cc. of milk to a 20 cc. test tube containing one cc. of a 1-20,000 solution of methylene blue. All of the tubes were incubated in a water bath at a temperature of 37½ degrees Centigrade, until reduction took place. The temperature of the water bath did not vary more than two degrees at any time. All tubes were examined at fifteen minute intervals, so as to determine the exact end point. Since each sample of milk was relatively fresh it did not take long to pass through the visible zone of reduction. The average time required for decolorization

to take place in the two duplicate tubes was reguarded as the reduction time.

The plate count was made according to the procedure outlined by the American Public Health Association for milk analyses. Dilutions were made so that no plate would contain less than 40 or more than 400 colonies.

The microscopical count was used on most of the milk samples to secure a general idea of the bacterial flora, but no actual counts were recorded since such counts would not be in harmony with the plate count. This test also enabled the detection of an excessive number of leucocytes in the milk.

Results Secured-- All of the following samples were collected from November 8 to March 28, 1935, and were analyzed according to the procedure outlined above.

Table No. 3 Analysis of milk produced in the ordinary way

Sample Number	1	2	3	4	5	6	7	8	9	10
Vol. Milk Used	170	170	170	170	170	170	170	170	170	170
Total Gas in CC.	7.57	8.50	5.44	7.72	7.65	7.65	8.00	8.11	9.43	7.59
Free O_2 in CC.	.62	.69	.62	.04	.85	.92	1.00	.15	.85	1.08
% O_2 in Total Gas	8.16	8.18	11.39	.49	11.11	12.00	12.50	1.90	9.01	14.28
Free CO_2 in CC.	4.79	4.87	2.93	5.74	4.17	4.21	4.38	5.48	5.56	4.01
% CO_2 in Total Gas	63.26	57.27	53.88	74.47	54.54	55.00	54.80	67.61	59.00	53.06
Res. Gas in CC.	2.16	2.94	1.89	1.94	2.63	2.52	2.62	2.48	3.02	2.48
% Res. Gas in Total Gas	28.57	34.55	34.81	25.02	34.35	33.00	32.70	30.49	31.99	32.67
Meth. Blue Red Test - Hrs.:Min.	1:55	2:25	3:00	:25	3:20	6:30	6:00	:15	7:15	8:30
Plate Count	Contaminated			69,000	21,000	29,000	6,500	265,000	4,250	1,800

Table No. 4 Gas analyses of milk produced in the ordinary way

Sample Number	11	12	13	14	15	16	17	18	19	20
Vol. Milk Used	170	170	170	170	170	170	170	170	170	170
Total Gas in CC.	9.27	9.10	9.20	9.40	8.20	8.20	6.80	6.60	7.40	7.10
Free O_2 in CC.	.92	.60	1.00	1.00	1.00	.90	1.00	.70	.60	.80
% O_2 in Total Gas	10.00	6.59	10.86	10.63	12.19	10.97	14.76	10.60	8.10	11.26
Free CO_2 in CC.	5.41	6.10	5.00	5.60	4.70	5.20	2.90	3.80	4.60	4.10
% CO_2 in Total Gas	58.33	67.03	54.34	59.57	57.31	63.41	42.64	57.57	62.16	57.74
Res. Gas in CC.	2.94	2.40	3.20	2.80	2.50	2.10	2.90	2.10	2.20	2.20
% Res. Gas in Total Gas	31.67	26.37	34.78	29.80	30.50	25.62	42.64	31.82	29.74	31.00
Meth. Blue Red Test - Hrs.:Min.	3:45	2:30	3:10	9:45	7:30	6:45	4:45	11:10	4:90	7:00
Plate Count	3,000	3,250	248,000	3,225	Cont'd.		36,000	22,000	94,000	2,100

Table No. 5 Gas analyses of milk produced in the ordinary way

Sample Number	21	22	23	24	25	26	27	28	29	30
Vol. Milk Used	170	170	170	170	170	170	170	170	170	170
Total Gas in CC.	7.90	5.85	4.40	6.46	6.46	6.60	6.50	6.88	6.70	7.00
Free O_2 in CC.	1.60	.80	.80	.92	.92	.15	.20	1.10	1.00	1.00
% O_2 in Total Gas	20.25	13.67	14.81	14.24	14.24	2.34	3.07	16.03	15.00	14.28
Free CO_2 in CC.	2.90	2.65	1.40	3.25	3.50	4.40	4.10	3.28	3.45	3.60
% CO_2 in Total Gas	36.70	45.30	25.92	50.31	54.17	68.75	63.07	46.42	51.50	51.43
Res. Gas in CC.	3.40	2.40	2.20	2.04	2.04	2.05	2.20	2.50	2.25	2.40
% Res. Gas in Total Gas	43.05	41.03	59.27	35.45	31.59	28.91	33.86	37.55	33.50	34.29
Meth. Blue Red Test - Hrs.:Min.	5:00	8:00	7:10	9:00	8:40	:15	:20	9:40	5:40	4:45
Plate Count	71,000	93,400	39,000	8,725	7,250	43,000	9,000	5,250	63,000	30,660

Table No. 6 Gas analyses of milk produced in the ordinary way

Sample Number	31	32	33	34	35	36	37	38	39	40
Vol. Milk Used	170	170	170	170	170	170	170	170	170	170
Total Gas in CC.	6.74	6.57	6.65	6.81	4.50	3.50	3.00	4.30	5.00	5.15
Free O_2 in CC.	.96	1.06	.37	.83	.80	1.00	.15	.40	1.00	.88
% O_2 in Total Gas	14.47	16.20	5.55	12.19	17.77	28.57	5.00	9.30	20.00	17.24
Free CO_2 in CC.	3.72	3.02	4.61	3.31	1.80	.50	1.40	2.90	1.90	2.48
% CO_2 in Total Gas	55.26	45.92	69.44	48.60	40.00	14.28	46.66	67.44	38.00	48.27
Res. Gas in CC.	2.06	2.49	1.67	2.67	1.90	2.00	1.45	1.00	2.10	1.79
% Res. Gas in Total Gas	30.27	37.88	25.00	39.21	42.23	57.15	48.34	23.26	42.00	34.49
Meth. Blue Red Test - Hrs.:Min.	9:30	8:50	7:45	8:15	8:10	7:05	:10	5:10	7:00	8:30
Plate Count	5,900	6,170	750	13,050	5,375	45,000	980,000	852,000	189,000	10,000

Table No. 7 Gas analyses of milk produced in the ordinary way

Sample Number:	41	42	43	44	45	46	47	48	49	50
Vol. Milk Used	170	170	170	170	170	170	170	170	170	170
Total Gas in CC.	5.80	5.70	5.70	6.04	6.60	7.70	7.40	6.70	7.10	7.80
Free O_2 in CC.	.58	.83	1.00	.74	1.00	1.00	1.10	1.10	.40	1.10
% O_2 in Total Gas	10.00	14.49	17.54	12.32	15.15	14.28	14.86	16.42	5.63	14.10
Free CO_2 in CC.	3.15	2.89	2.50	3.31	3.00	4.30	3.90	3.20	3.40	4.20
% CO_2 in Total Gas	54.28	50.72	43.86	54.80	45.45	55.84	52.70	47.76	47.88	53.97
Res. Gas in CC.	2.07	1.98	2.20	1.99	2.60	2.40	2.40	2.40	3.30	2.50
% Res. Gas in Total Gas	35.72	34.79	38.60	32.88	39.40	29.88	32.44	35.82	46.49	31.93
Meth. Blue Red Test - Hrs.:Min.	4:00	3:30	8:00	5:40	8:50	9:00	9:45	8:30	10:35	2:35
Plate Count	340,000	900,000	3,970	8,050	3,420	31,000	4,850	3,850	20,000	72,000

Table No. 8 Gas analyses of milk produced in the ordinary way

Sample Number:	51	52	53	54	55	56	57	58	59	60
Vol. Milk Used	170	170	170	170	170	170	170	170	170	170
Total Gas in CC.	7,70	7.20	7.00	7.90	7.10	6.40	6.40	6.20	7.80	7.82
Free O_2 in CC.	1.10	0.90	.60	.70	1.05	.70	.80	1.00	.17	.19
% O_2 in Total Gas	14.10	12.50	8.57	8.86	14.78	10.94	12.50	16.13	2.27	2.50
Free CO_2 in CC.	4.30	3.20	3.00	4.90	3.40	3.60	3.40	2.60	4.96	5.47
% CO_2 in Total Gas	55.12	44.44	42.85	62.02	47.88	56.25	53.12	41.93	63.63	70.00
Res. Gas in CC.	2.40	3.10	3.40	2.30	2.65	2.10	2.20	2.60	2.66	2.16
% Res. Gas in Total Gas	30.78	43.06	48.58	29.12	37.34	32.81	34.38	41.94	34.10	27.50
Meth. Blue Red Test - Hrs.:Min.	8:30	8:30	2:25	1:15	8:00	13:20	13:15	13:00	:15	:45
Plate Count	22,825	7,000	13,900	570,000	4,700	5,375	2,120	2,550	354,000	2,000

Table No. 9 Gas analyses of milk produced in the ordinary way

Sample Number:	61	62	63	64	65	66	67	68	69	70
Vol. Milk Used	170	170	170	170	170	170	170	170	170	170
Total Gas in CC.	7.44	7.32	7.90	6.00	6.00	5.20	6.20	6.40	6.50	6.00
Free O_2 in CC.	.84	.84	1.19	1.20	.40	.05	1.00	1.20	.80	1.10
% O_2 in Total Gas	11.29	11.47	15.15	20.00	6.66	15.83	16.13	18.75	12.30	18.33
Free CO_2 in CC.	4.20	3.48	3.83	1.92	2.40	2.80	2.60	2.80	3.50	2.40
% CO_2 in Total Gas	56.45	47.54	48.48	32.00	40.00	46.66	41.93	43.75	53.84	40.00
Res. Gas in CC.	2.40	3.00	2.88	2.88	3.20	2.35	2.60	2.40	2.20	2.50
% Res. Gas in Total Gas	32.26	40.99	36.37	48.00	53.34	37.51	41.94	37.51	33.84	41.66
Meth. Blue Red.Test - Hrs.:Min.	6:00	8:30	2:00	8:55	10:10	7:15	8:30	10:00	10:05	7:30
Plate Count	9,730	10,500	52,350	2,475	1,175	8,400	4,550	4,925	57,000	2,850

Table No. 10 Gas analyses of milk produced in the ordinary way

Sample Number:	71	72	73	74	75	76	77	78	79	80
Vol. Milk Used	170	170	170	170	170	170	170	170	170	170
Total Gas in CC.	8.20	8.00	8.00	8.20	8.60	8.50	8.29	7.60	7.60	7.80
Free O_2 in CC.	.80	1.00	.30	.30	.40	1.30	.98	1.00	.90	1.20
% O_2 in Total Gas	9.75	12.50	3.75	3.66	4.65	15.29	11.84	13.15	11.84	15.38
Free CO_2 in CC.	5.00	4.40	5.10	5.70	6.00	3.90	4.25	4.10	3.90	3.50
% CO_2 in Total Gas	60.97	55.00	63.75	69.51	69.76	45.88	51.31	53.94	51.31	44.87
Res. Gas in CC.	2.40	2.60	2.60	2.20	2.20	3.30	3.06	2.50	2.80	2.30
% Res. Gas in Total Gas	29.28	32.50	32.50	26.83	25.59	38.83	36.85	32.91	36.85	39.75
Meth. Blue Red.Test - Hrs.:Min.	11:15	4:45	:20	:20	:20	8:35	7:00	7:45	8:00	13:05
Plate Count	67,000	107,000	3,465,000	25,800	1,260,000	6,425	3,470	20,720	3,470	14,650

Table No. 11 Gas analyses of milk produced in the ordinary way

Sample Number:	81	82	83	84	85	86	87	88	89	90
Vol. Milk Used	170	170	170	170	170	170	170	170	170	170
Total Gas in CC.	7.40	7.40	5.04	5.20	5.54	5.01	5.16	4.80	5.70	6.00
Free O_2 in CC.	1.00	.90	.34	.10	.18	.68	.76	.10	.70	1.10
% O_2 in Total Gas	13.51	12.16	6.66	11.92	3.33	13.67	14.81	2.08	12.28	18.33
Free CO_2 in CC.	4.20	4.20	2.05	3.00	3.32	2.27	1.34	3.00	2.40	2.40
% CO_2 in Total Gas	56.75	56.75	40.00	57.69	60.00	45.30	25.92	62.50	42.10	40.00
Res. Gas in CC.	2.20	2.30	2.75	2.10	2.04	2.06	3.06	1.70	2.60	2.50
% Res. Gas in Total Gas	29.75	31.11	53.34	40.39	36.67	41.03	59.27	35.42	45.62	41.67
Meth. Blue Red.Test - Hrs.:Min.	13.00	11.10	10.10	:10	10:15	7:00	6:50	:05	6:00	7:45
Plate Count	10,420	6,220	1,175	579,000	75,500	93,400	39,000	201,000	25,370	2,850

Table No. 12 Gas analyses of milk produced in the ordinary way

Sample Number:	91	92	93	94	95	96	97	98	99	100
Vol. Milk Used	170	170	170	170	170	170	170	170	170	170
Total Gas in CC.	5.80	5.50	5.20	6.00	6.40	6.40	6.60	6.50	6.80	6.60
Free O_2 in CC.	.10	1.00	.40	.35	1.00	.60	.90	.10	.85	1.00
% O_2 in total gas	1.72	18.18	7.69	5.83	15.62	9.37	13.63	1.51	12.50	15.15
Free CO_2 in CC.	3.50	2.00	2.40	3.40	2.90	3.60	3.50	4.10	3.90	3.20
% CO_2 in Total Gas	60.34	36.36	46.15	56.66	45.31	56.25	53.03	62.11	57.35	48.78
Res. Gas in CC.	2.20	2.50	2.40	2.25	2.50	2.20	2.20	2.30	2.05	2.40
% Res. Gas in Total Gas	37.94	45.46	46.16	37.51	39.07	34.38	33.34	36.38	30.15	36.07
Meth. Blue Red.Test - Hrs.:Min.	:20	7:30	3:20	2:55	9:30	9:40	6:30	:30	10:00	9:15
Plate Count	630,000	166,000	1,440,000	1,095,000	4,000	63,500	780,000	1,000,000	3,700	4,120

Table No. 13 Gas analyses of milk produced in the ordinary way

Sample Number:	101	102	103	104	105	106	107	108	109	110
Vol. Milk Used	170	170	170	170	170	170	170	170	170	170
Total Gas in CC.	6.70	7.00	7.20	7.00	7.00	9.00	8.75	9.00	8.20	7.90
Free O_2 in CC.	.80	.15	.40	.50	.90	.85	.30	.90	.90	1.60
% O_2 in Total Gas	11.94	2.14	5.55	7.14	12.86	9.44	3.42	10.00	10.97	20.25
Free CO_2 in CC.	3.70	4.30	4.10	4.30	3.30	5.40	6.05	5.90	5.20	2.90
% CO_2 in Total Gas	55.22	60.14	56.94	61.43	47.14	60.00	69.71	65.55	63.41	36.70
Res. Gas in CC.	2.20	2.55	2.70	2.20	2.80	2.75	2.40	2.20	2.10	3.40
% Res. Gas in Total Gas	38.84	37.72	37.51	31.43	40.00	30.56	26.87	24.45	25.62	43.05
Meth. Blue Red.Test - Hrs.:Min.	12:45	:15	1:25	:50	8:00	6:30	:35	8:20	7:45	6:15
Plate Count	89,000	3,690,000	1,071,000	71,500	85,000	28,500	39,000	10,250	14,850	14,900

Table No. 14 Gas Analyses of milk produced in the ordinary way

Sample Number:	111	112	113	114	115	116
Vol. Milk Used	170	170	170	170	170	170
Total Gas in CC.	7.10	7.40	7.20	7.30	8.00	7.80
Free O_2 in CC.	.80	.90	1.10	.90	1.00	.15
% O_2 in Total Gas	11.26	12.16	15.27	12.33	12.50	1.92
Free CO_2 in CC.	4.10	4.30	3.70	4.40	4.40	5.00
% CO_2 in Total Gas	57.74	56.75	51.39	50.68	55.00	64.10
Res. Gas in CC.	2.20	2.30	2.40	2.70	2.60	2.65
% Res Gas in Total Gas	31.00	31.18	33.34	36.99	32.50	33.98
Meth. Blue Red.Test - Hrs.:Min.	8:00	11:10	5:50	12.15	11:45	:05
Plate Count	2,100	6,220	6,150	14,135	857,000	86,000

One hundred and sixteen samples of milk were collected as it was brought into the V. P. I. Creamery, and analyzed immediately. The percentages given above show that there is considerable variation in the per cent of oxygen in milk when it is produced in the ordinary way. Provided that all of the other factors in the methylene blue reduction test are constant, except the per cent of oxygen, there would be a variation corresponding to the amount of dissolved oxygen in milk. The higher the percentage of dissolved oxygen the longer the reduction time, when methylene blue is used as an indicator.

The highest per cent of oxygen found in the 116 samples was 20.50 and the lowest was .49. The average per cent of oxygen for all of the samples was 11.24. It was found that this percentage could be increased by aerating over a cooler or by pouring slowly from one flask to another. An average of .76 cubic centimeters of oxygen (or .45% by volume) was obtained from the group of samples when 170 cc. of milk was used for the analyses.

With several exceptions----which will be explained in a subsequent paragraph----the milk which was first to lose its color was highest in bacterial numbers and low in dissolved oxygen. The exception to this would normally be anticipated and would account for variation in the methylene blue reduction test.

In the majority of analyses, the approximate reduction time and also the number of bacteria could be estimated by a knowledge of the dissolved oxygen content of the milk. In every case, the samples of milk which were low in dissolved oxygen were first to lose the color imparted by the addition of methylene blue. After the amount of oxygen contained in the milk was reduced to a certain low percentage, the methylene blue reduced to its leucobase. By having a thorough knowledge of the dissolved oxygen content of milk it is thought that its keeping quality could be estimated with a fair degree of accuracy.

The smallest volume of oxygen obtained from any 170 cc. sample of milk was .05 cc. and the largest volume was 1.20 cc. All samples of milk which had a high dissolved oxygen content were found to retain the color of the dye for a longer period of time than milk with a low dissolved oxygen content.

The gas analyses reveals the percentage of carbon dioxide to be quite a variable factor. In the 116 samples there was an average of 52.77 per cent of carbon dioxide. The highest percentage of carbon dioxide was 69.71 and the lowest was 25.92. In the entire group of milk samples the average volume of carbon dioxide was 3.605 cubic centimeters in 170 cc. As previously shown by experiments, the interchange of carbon dioxide and

oxygen is facilitated by thorough aeration. When the milk is drawn from the cow it is relatively high in carbon dioxide and on aeration this amount is reduced by interchanging with atmospheric oxygen.

The residual gas existed in the samples of market milk to the extent of 35.99 per cent of the total gas, which is an average for the 116 samples. No attempt was made to make a quantitative or a qualitative analysis of the residual gas. The author was primarily interested in the oxygen content and its variation. It was found that several samples were low in bacterial numbers and low in dissolved oxygen, hence they reduced the dye to its leucobase in a short period of time, thereby erroneously indicating a milk of poor quality. There is a distinct variation in the type of bacteria which dominate the flora and it is known that certain species of bacteria differ in their ability to consume oxygen. With this fact in mind, the oxygen-consuming power of certain species would be of no value in determining the reduction time of methylene blue.

The amount of available oxygen in milk for oxidation by bacteria is an important question in milk sanitation; especially, to the extent to which the oxygen content may be reduced before putrefaction sets in. As long as there is sufficient oxygen the bacteria

will carry the organic matter to completion, but when the oxygen is reduced to a certain low percentage, putrefaction sets in and is always accompanied by foul odors. The aeration of milk directly increases the amount of dissolved oxygen, which in turn plays a part in delaying putrefaction.

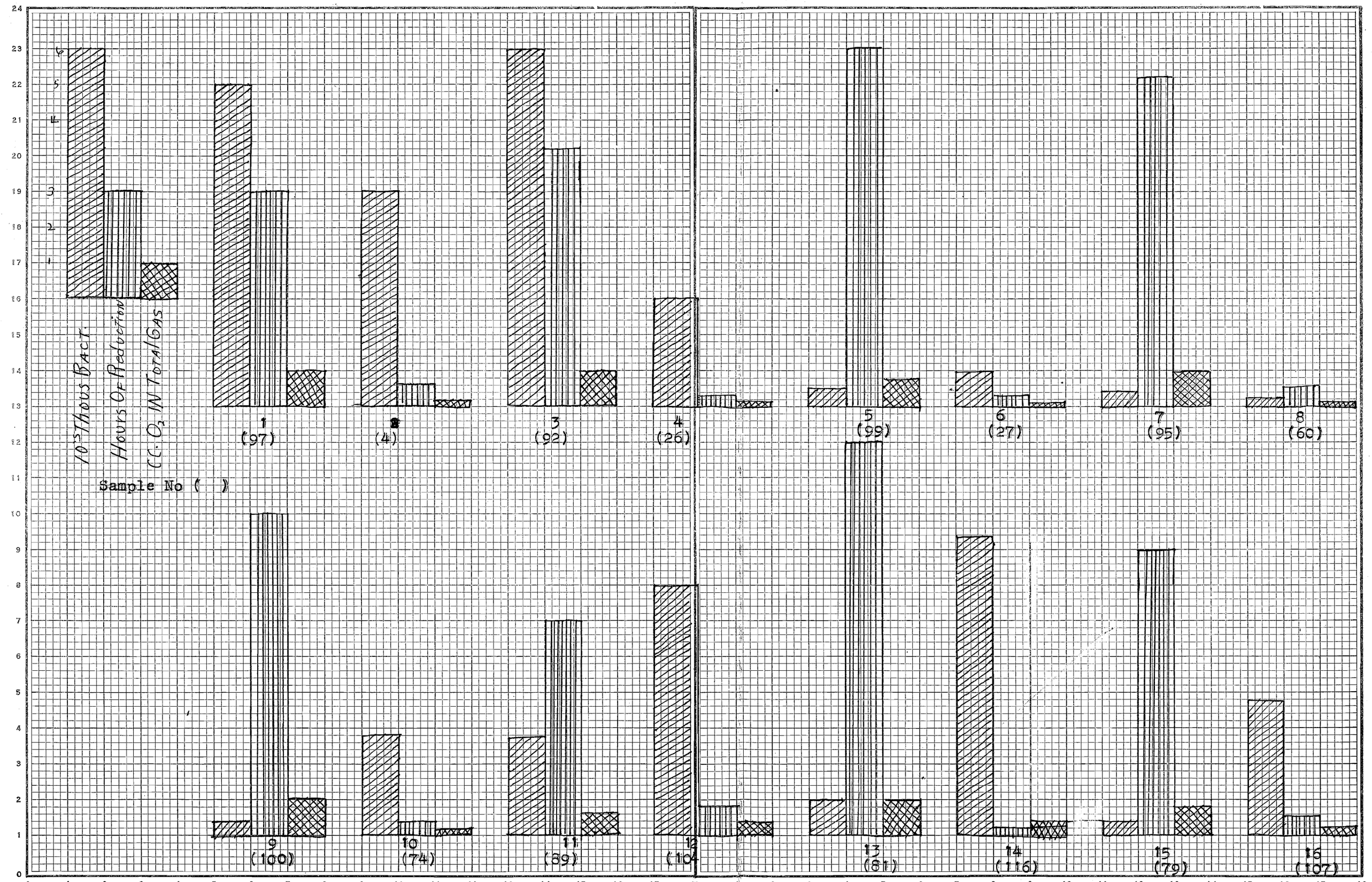
The number of bacteria, period of methylene blue reduction, and the amount of dissolved oxygen found in sixteen samples of milk is graphically represented on page 38. The odd numbered graphs represent the samples of milk having a normal reduction time and the even numbered represent the samples of milk having an abnormal reduction time, when methylene blue is used as an indicator.

An introspection of the graphs reveals the fact that the number of bacteria is a fluctuating and uncontrollable factor. However, the period of methylene blue reduction is in direct proportion to the amount of dissolved oxygen. The reduction time of methylene blue and the amount of dissolved oxygen are very consistent regardless of whether they represent samples of milk having a normal or abnormal period of reduction (in proportion to the number of bacteria).

The reduction time of methylene blue shown in the even numbered graphs is not representative of

the bacterial count. According to the normal findings in the test, the samples of milk having a low bacterial count should have a relatively long period of reduction, Eight discrepancies were noted in the 116 samples of milk studied.

Each graph on page 38 will show that the period of reduction and the amount of dissolved oxygen exist in a definite and consistent ratio.



Graph No. 1 A graphical representation of milk having normal and abnormal reduction periods.

The following table gives the analyses of eight samples of milk, which showed inaccurate methylene blue reduction tests.

Table No. 14 Analyses of milk which failed to coincide when the Methylene blue reduction test and plate count was applied

Sample Number:	4	26	27	60	74	104	116	107
Vol. Milk Used	170	170	170	170	170	170	170	170
Total Gas in CC.	7.72	6.40	6.50	7.82	8.20	7.00	7.80	8.75
Free O_2 in CC.	.04	.15	.20	.19	.30	.50	.15	.30
% O_2 in Total Gas	.49	2.34	3.07	2.50	3.66	7.14	1.92	3.42
Free CO_2 in CC.	5.74	4.40	4.10	5.47	5.70	4.30	5.00	6.05
% CO_2 in Total Gas	74.47	68.75	63.07	70.00	69.51	61.43	64.10	69.71
Res. Gas in CC.	1.94	1.85	2.20	2.16	2.20	2.20	2.65	2.40
% Res. Gas in Total Gas	25.02	28.91	33.86	27.50	26.83	31.43	33.98	26.87
Meth. Blue Red.Test -Hrs.:Min!	:25	:15	:20	:45	:20	:50	:05	:35
Plate Count	59,000	43,000	9,000	2,000	25,800	71,500	86,000	39,000

Sample number 27 reduced methylene blue to its leucobase in twenty minutes, but only contained 9,000 bacteria per cc. of milk. If the milk contained the normal amount of oxygen incorporated by aeration it should not reduce a milk containing 9,000 bacteria under seven hours.

In the preceding table we find that the reduction time of each sample should normally correspond to a bacterial count of 1,000,000 per cc. In reality, the bacterial count never exceeded 26,000 bacteria per cc. Every sample listed in Table No. 14 showed a very low percentage of oxygen, which accounts for the quick reduction. It only required a few minutes for the bacteria to consume the oxygen and reduce methylene blue to its leucobase.

THE EFFECT OF METHODS OF SAMPLING AND MIXING MILK AND DYE ON REDUCTION TIME

The mixing of milk and dye has received considerable attention by many operators of the methylene blue reduction test. Several different methods of mixing both dye and milk were studied to determine which method gives the most consistent results. Most workers have

found that a large percentage of the variation in the test can be attributed to the improper mixing or sampling of dye and milk.

Before drawing a sample of milk from a container it should be thoroughly mixed to get a uniform distribution of the butterfat and accompanying bacteria. It has been shown by Anderson (19) that the gravity cream contains fifteen times as many bacteria as the bottom milk, therefore it is quite important to get a uniform distribution of the butterfat before a sample is taken. The most accurate results have been obtained when a sterile pipette was used to transfer the milk to a test tube.

In order to obtain a uniform amount of oxygen in the milk it is indispensable to wash the dissolved oxygen from the milk, or make an effort to incorporate the maximum amount of oxygen. It is rather expensive and impracticable to wash each sample of milk with an inert gas, such as hydrogen, nitrogen, or carbon dioxide.

Since it is very difficult to reduce the oxygen content of milk, the simplest method would be to incorporate the maximum amount of oxygen by a thorough aeration process.

The Effect of Excess Aeration on the Reduction of Methylene Blue-- The simplest method of aeration consists of inverting one glass funnel into the top of another, and then slowly pouring the milk on the upper funnel so as to let it flow in a thin film over the entire surface of the funnel. This procedure is described on page 21, and it should be repeated several times. Five samples of milk were analyzed before and after aeration. In each case there was a distinct increase in the amount of dissolved oxygen and also an increase in the reduction time, due to aeration. Analyses of the five samples are given in the table below.

Table No. 15 Effect of aeration on the reduction time of methylene blue when added to milk

Sample Number:	1	2	3	4	5	6
% oxygen before aeration	10.10	9.05	9.03	11.43	12.08	12.30
% O_2 in total gas after aeration	15.62	15.15	14.81	15.29	15.38	15.83
Meth. Blue Red. before aeration Hrs.:Min.	6:10	6:00	5,10	7:00	8:45	5.25
Meth. Blue Red. Test after aerat'n, Hrs:Min	9:30	9:15	6:35	8:35	13.05	7:15
No. of bact per cc.	7,220	4,120	21,000	6,525	14,650	8,400

In sample five the reduction time increased (4:20) four hours and twenty minutes, because of aeration, and in sample three the reduction time only increased (1:25) one hour and twenty-five minutes because of aeration. The figures in the table above show conclusively that there is

a necessity of further aeration.

Suggested Method of Mixing Milk-- In order to eliminate any variation or inaccuracies in the test, it is imperative that each sample be brought to an oxygen equilibrium. If uniformity is to be obtained the same aeration process must be given to each sample prior to setting up the methylene blue reduction test. It is very evident that some samples of milk approach oxygen equilibrium closer than others. In setting up the methylene blue reduction test, each 10 cc. sample of aerated milk should be placed in a twenty cubic centimeter test tube of uniform size to obtain the same exposure to atmospheric air.

Method of Mixing Methylene Blue

Age of Methylene Blue Solution-- In order to determine the effect of age on solution of methylene blue, tests were made with freshly prepared solutions of the dye and also with solutions seven months old. The results obtained from both were almost identical. From this study it appears as though the age of the solution would have little if any effect in the practical application of the test.

A number of investigators advocate the use of freshly prepared solutions of methylene blue. The

probable explanation for this lies in the fact that old samples are more subject to contamination if care is not taken to seal the mouth of the container.

Effect of Concentration of the Dye-- A considerable number of trials have been made to determine the effect of dye on the reduction time and the concentration best adapted for employment in the test. The main purpose of this investigation is to determine whether a concentration of 1-20,000 solution is sufficient or inadequate when added to a 10 cc. sample of milk.

This problem was approached by comparing a 1-20,000 solution of methylene blue with both a stronger and a weaker solution. When the solution of methylene blue is too strong it produces an antiseptic effect and the activity of the organisms are decreased.

In an effort to determine the best concentration of methylene blue to employ in the test, the author has compared three different dilutions of dye on the same sample of milk.

Table No. 15 tabulates the results.

Table No. 15 Determining the Effect of Dye Concentration on the reduction of methylene blue when added to milk

Sample No.	No. of Bact. per cc. of milk	1-20,000 Sol.	1-15,000 Sol.	1-30,000 Sol.
1	30,660	4:45	4:55	5:35
2	13,050	8:15	8:40	8:00
3	248,000	3:10	3:25	2:55
4	46,000	5:45	5:45	5:35
5	63,000	5:40	5:55	5:30

In the above table we find that the 1-15,000 solution of methylene blue when added to milk retained the blue color over a longer period, with one exception, than did the 1-20,000 solution of methylene blue. A fresh sample of milk was always used, since it facilitated a rapid passage through the zone of visible reduction. The end point was fairly distinct in each case. The 1-30,000 solution of methylene blue was always quicker than the 1-20,000 solution to pass through the zone of visible reduction, and the reduction time was always shorter in each case. A probable explanation for the longer period of reduction, in the case of the more concentrated solution, lies in the fact that methylene blue acts as an antiseptic when in strong solutions and decreases the activity of bacteria. With a decrease in the activity of bacteria there is a corresponding decrease in the rate of oxygen consumption.

Oxygen in the Methylene Blue Solution-- It occurred to the author that there is a possibility of introducing dissolved oxygen when adding a solution of methylene blue to milk. In view of this possibility, several 1-20,000 solutions of methylene blue were prepared and analyzed for the per cent of oxygen, in the same way as for milk. A large volume of methylene blue was prepared and divided into three parts. The first two samples were autoclaved for ten minutes and allowed to cool. The third sample was thoroughly aerated by applying the funnel method used in aerating milk.

Results Secured-- The results obtained from the three samples are as follows.

Table No. 16 Analyses of the gas content of methylene blue solutions

Sample No.	Vol. Sol. used	CC. gas	CC. oxygen	% oxygen	CC. CO_2	% CO_2	CC. Red. gas	% Res. gas
1	180	4.00	.80	20.00	.20	5.00	3.00	75.00
2	180	4.20	.80	19.05	.20	7.40	1.70	73.55
3	180	2.70	1.20	28.57	.20	4.76	2.80	66.67

According to the results obtained in Table No. 16 it is possible to incorporate additional oxygen in a dye solution when it is subject to aeration. Sample No. 3 gave an increase of 8.57 per cent oxygen over the unaerated sample. It appears that this amount would

certainly be of significance in lengthening the reduction time when added to milk. If uniformity of results are to be obtained it would seem advisable to thoroughly aerate the methylene blue solution before adding it to milk. By properly preparing the dye and following the above suggestions it is believed that a large percentage of variation, due to mixing of dye, can be eliminated.

CONTROLLING REDUCTION TIME BY THE ADDITION OF SALTS AND OTHER SUBSTANCES

Since the reduction of methylene blue involves a transfer of hydrogen it seems probable that many compounds act in the capacity of hydrogen donators. If there is not sufficient hydrogen present the methylene blue will not be reduced, despite the fact that all of the oxygen has been consumed. Provided an adequate supply of hydrogen is available, the methylene blue will not act as an acceptor for the hydrogen and will undergo reduction, when the oxygen has been consumed by the bacteria.

In order to be assured that there is sufficient hydrogen in the milk to unite with methylene blue, the addition of a hydrogen donator was thought to be of

value. If some inexpensive salt could be added to milk to shorten the reduction time the results could be secured much quicker, thereby rendering the test more practicable.

In this experiment a large number of salts were added to milk in an endeavor to reduce the reduction time when methylene blue is added as an indicator.

Procedure-- The procedure for the experiment will consist in adding methylene blue to test tubes containing various salts. One tenth (.1) gram of each salt is added to a group of clean test tubes, in duplicate. Each test tube is then plugged with cotton and sterilized in a dry oven at a temperature of 175 degrees Centigrade for one hour. This is done in order to eliminate any contamination which may come from the salts which are selected. Ten cc. of milk and one cc. of a 1-20,000 solution of methylene blue is added to each test tube. All tubes were thoroughly shaken to mix the methylene blue and the small quantity of salt.

Since the salt was added to the test tubes in duplicate, an effort was made to keep both tubes together in the incubator. All tubes were placed in a water bath at a temperature of 37 degrees Centigrade until reduction took place. The average time required for the blue color

to disappear from each set of test tubes, is taken as the reduction time. The coloring and rapidity of reduction in various parts of each test tube was noted and considered in the final analyses.

In addition to the inorganic salts, a small chip of beef (.1 gram), and one gram of an Irish potato was added to several test tubes in duplicate. It is thought that the animal and vegetable tissue were more instrumental in absorbing oxygen than in donating hydrogen.

Results Secured-- Results obtained from the four samples of milk are given in the following table.

Table No. 17 Effect of salts and other substances on the reduction of methylene blue in Sample No. 4

Name of substance	Wt. or Vol. used	Red. Time- Hrs.: Min.	Remarks
Potassium Iodide	.1 gr	6:45	Required long period to discolor
Sodium Chloride	"	7:10	Formed a reddish layer in bottom of tube
Citric Acid	"	10:15	Retained blue color longer than check
Calcium Carbonate	"	9:10	Fell rapidly thru zone of reduction
Potassium Phosphate	"	7:25	Dark blue color in bottom of tube
Calcium Sulphate	"	9:00	Reduction normal
Dextrose	"	7:35	Fairly uniform reduction

(Table No. 17 cont'd)

Name of substance	Wt. or Vol. used	Red. Time- Hrs.: Min.	Remarks
Potassium Chloride	.1 gr	7:15	Long period to pass thru zone of red.
Amm. Citrate	"	8:05	Imparted dark tinge to milk
Ethyl Alcohol	.2 cc	8:20	Aided slightly in fat distribution
Mineral Oil	1.5cc.	8:50	Acted as a seal for the tube
Irish Potato	.1 gr	7:45	Very good oxygen absorbent
Check or untreated sample		10:10	Reduction normal

Table No. 18 Effect of salts and other substances on the reduction of methylene blue when added to milk sample No. 6

Name of substance	Wt. or Vol.	Red. T Hr/Min	Remarks
Irish Potato	.1 gr.	9:35	Reduction normal
Beef	"	2:45	Reduction occurred in bottom of tube first
Lactose	"	10:45	Reduction normal
Potassium Iodide	"	9:30	Reduction normal
Potassium Phosphate	"	10:35	Required long period to pass thru zone of Red.
Sodium Silicate	"	10:05	Milk turned red in bottom of tube
Sodium Carbonate	"	9:30	Reduction very uniform
Ethyl Alcohol	.1 cc.	9:50	Reduction normal

(Table No. 18 cont'd)

Name of substance	Wt. or Vol.	Red.T. Hrs.: Min.	Remarks
Mineral Oil	1.5 cc	10:15	Acted in the capacity to keep out the air
Check or untreated sample	.1 gr	10:35	Reduction normal

Table No. 19 Effect of salts and other substances on the Red. of meth. blue in milk sample #8

Name of substance	Wt. or Vol.	Red. T. Hr/Min.	Remarks
Magnesium Sulphate	.1 gr.	4:45	Slow to pass thru the zone of reduction
Dextrose	"	3:20	Rather rapid to pass thru zone of red.
Lactose	"	5:15	Reduction normal
Potass. Sulphate	"	3:05	Dark blue in bottom of tube
Sodium Chloride	"	5:45	Reduction normal
Calcium Sulphate	"	5:30	Reduction normal
Potass. Iodide	"	4:05	Reduction normal
Citric Acid	"		The acid coagulated the milk - blue color persisted
Sod. Carbonate	"	5:00	Reduction fairly uniform
Beef	"	2:55	The milk first lost its color at the bottom of tube
Irish Potato	"	4:55	Reduction normal
Check or untreated	"	6:15	Reduction normal

Almost every salt which was added to milk in the above tables reduced methylene blue before the check tubes. This is an indication that the salts did not inhibit the growth of bacteria, but rather acted in the capacity of a hydrogen donator.

The small piece of beef was very effective in reducing the methylene blue to its leucobase. This was facilitated by the absorption of dissolved oxygen as well as the fostering of bacterial growth. The vegetable tissue brought about quicker reduction than the untreated sample, but was quite inferior to beef as an oxygen reducing agent. In every case the small piece of beef gave a quicker reduction, which was very constant throughout the experiment.

When dextrose was added to milk it greatly increased the rapidity of reduction and brought about very uniform end-points. In each experiment the milk passed rapidly through the visible zone of reduction. When the same amount of lactose was added to milk there were no changes of significance. In one experiment the lactose delayed reduction, and in another it shortened the reduction time slightly. In neither experiment did it show as promising results as dextrose.

An introspection of the results obtained by the addition of salts reveals the fact that potassium

iodide is much superior to the other salts as a reducing agent. The small quantity of potassium iodide gave an appreciable decrease in the reduction time of methylene blue when added to milk.

Where results from the methylene blue reduction test are to be obtained quickly, it is believed that a uniform addition of potassium iodide or dextrose would maintain the delicacy of the test and also enable the results to be obtained much sooner than by following the ordinary procedure. It appears that .1 Gram would be the most desirable quantity to add to 10 cc. of milk.

It seems very pertinent that there is a slight advantage in sealing the test tube with one and a half cc. of mineral oil, since it prevents the seepage of atmospheric oxygen into the milk.

THE EFFECT OF METHYLENE BLUE ON THE GROWTH CURVE OF BACTERIA

Adequate time was not available to allow the completion of this phase of the work, but the procedure and results are present despite the fact that no conclusions can be drawn.

It is well known that methylene blue inhibits the growth and activity of bacteria and that some are more strongly affected than others. The object of this experiment is to determine whether the strength of methylene blue, commonly employed in the test, is strong enough to have any harmful effect on the growth and multiplication of bacteria commonly occurring in milk.

If a 1-20,000 solution of methylene blue exerts a poisoning effect on the bacteria, it will cause a variation in the test and can only be corrected by using a less concentrated solution of methylene blue in the test. Provided that the dye exerts a detrimental effect on bacteria, it is quite probable that it would be more harmful to certain species of bacteria. Our first approach to the solution of the problem will consist in finding the effect of dye on the bacteria which frequent market milk.

Procedure-- The procedure consisted in dividing a well-mixed sample of market milk into two portions. Ten cc. of milk from the first portion was added to each of 10 sterile test tubes containing one cc. of a sterile 1-20,000 solution of methylene blue. Ten cc. of the second portion was added toeach of 10 sterile test tubes containing one cc. of sterile water. Both sets of test tubes were kept separate and incubated in a water bath at a temperature of 37 degrees Centigrade. One tube from each group was plated out in the proper dilution at half hour intervals until reduction took place. The plate counts were made according to the procedure outlined by the Americal Public Health Association. Before making dilutions for the plate count the milk in the test tubes were thoroughly shaken to distribute the butterfat and accompanying bacteria. Each dilution was plated out in duplicate and counted after 48 hours incubation at a temperature of 37 degrees Centigrade.

The sample of milk containing the sterile water will be considered as a check against the same sample of milk containing the dye. The purpose of adding the one cc. of sterile water to the 10 cc. of milk is to secure the equivalent dilution obtained by adding one cc. of the dye solution.

Results Secured-- The following tables will show the changes which occurred in the growth curve of bacteria in each sample of milk.

Table No. 20 Effect of methylene blue on the growth curve of bacteria

Sample No.	Interval of Incub.	Check Sample (with water)	Sample with Meth. Blue	Remarks
1	Initial	387,000	387,000	Mic.Count 4,200,000
1	30 Min.	343,000	319,000	
1	30 "	715,000	360,000	
1	30 "	1,073,000	1,250,000	
1	30 "	850,000	716,000	Reduction took place
1	30 "	1,170,000	983,330	

Table No. 21

2	Initial	contaminated		
2	30 Min.	contaminated		Dominating bacteria in milk were the Streptococcus lactis
2	30 "	2,255,000	2,120,000	
2	30 "	1,155,000	740,000	
2	30 "	1,310,000	940,000	

Table No. 22 Effect of methylene blue on the growth curve of bacteria

Sample No.	Interval of Incub.	Check Sample (with water)	Sample with Meth. Blue	Remarks
3	Initial	3,235,000	2,390,000	Microscopical count- 80,000,000
3	30 Min.	4,140,000	3,610,000	
3	30 "	5,450,000	5,315,000	
3	45 "	10,390,000	8,270,000	
3	30 "	14,530,000	13,440,000	
3	30 "	21,000,000	19,880,000	Reduction took place
3	30 "	34,650,000	29,980,000	

Table No. 23

4	Initial	1,450,000	1,450,000	Microscopical count- 10,990,800
4	30 Min.	1,725,000	3,900,000	
4	30 "	2,665,000	2,460,000	Dominating bacteria was Streps. and Staphs.
4	30 "	2,775,000	2,400,000	
4	30 "	3,870,000	5,530,000	
4	30 "	6,075,000	6,210,000	
4	30 "	7,465,000	7,670,000	
4	30 "	8,180,000	7,700,000	

Table No. 24 The effect of methylene blue on the growth curve of bacteria

Sample No.	Interval of Incub.	Check Sample (with water)	Sample with Meth. Blue	Remarks
5	Initial	1,333,335	1,333,335	Micros. count = 8,700,500
5	30 Min.	1,645,000	1,555,000	
5	30 "	3,975,000	1,810,000	
5	30 "	5,900,000	4,250,000	
5	30 "	7,120,000	6,290,000	
5	30 "	10,250,000	6,700,000	
5	30 "	15,100,000	14,900,000	Reduction took place

Table No. 25

6	Initial	3,900,000	3,900,000	Plate count at the beginning
6	30 Min.	7,400,000	4,950,000	
6	30 "	13,400,000	7,100,000	
6	45 "	26,750,000	14,400,000	
6	60 "	33,400,000	17,600,000	
6	30 "	47,500,000	19,750,000	
6	30 "	46,650,000	28,200,000	Reduction took place
6	60 "	47,200,000	27,200,000	

Table No. 26 The effect of methylene blue on the growth curve of bacteria

Sample No.	Interval of Incub.	Check Sample (with water)	Sample with Meth. Blue	Remarks
7	Initial	7,500,000	7,500,000	Micriscopical count- 12,100,000
7	30 Min.	13,500,000	11,500,000	
7	30 "	16,420,000	16,320,000	
7	45 "	22,000,000	21,000,000	
7	30 "	34,000,000	23,500,000	Reduction took place

In each of the above samples we find that methylene blue does prolong the lag phase, in bacterial numbers. The number of bacteria in milk containing methylene blue is much lower at the beginning of the incubation period, but as the bacteria become accustomed to their environment they grow more rapidly. However, until reduction takes place the number of bacteria in the check samples is always higher and is not affected as much at any time during the incubation period. From these studies it appears that a 1-20,000 solution of methylene blue when added to milk acts as an antiseptic and is detrimental to bacterial growth. After methylene blue had been reduced there was still a decrease in the number of bacteria in the sample of milk towhich methylene

blue had been added.

The dye seemed to exert a greater inhibitory effect on the bacteria in sample six than in any of the other samples, despite the fact that the same procedure was followed.

It is thought that methylene blue would prove more detrimental to certain species of bacteria than to others. Time did not permit an investigation with various species of bacteria, but this experiment appears to indicate that methylene blue produces an antiseptic action on the bacteria which frequent market milk.

Since a 1-20,000 solution of methylene blue when added to milk acts as an antiseptic, it seems that a 1-25,000 solution of methylene blue would not be as harmful to the bacterial flora of the milk. Only a few actual bacterial counts have been followed on the 1-25,000 solution of the dye, but all results seem to warrant such a change in the concentration of the dye solution.

CONCLUSIONS

After making a study of the methylene blue reduction test it is thought to be one of the simplest and most economical quality test for milk, provided that certain changes are made in the operation of the test. An introspection of the variations studied reveals the fact that there are certain factors which directly affect the accuracy of results, and it is thought that the following conclusions can be drawn.

1. The dissolved oxygen content of market milk fluctuates enough to cause a variation in the test.
2. The average per cent of oxygen in the total gases found in unexposed milk is 5.86.
3. The dissolved oxygen content of milk directly affects the reduction of methylene blue.
4. Excess aeration incorporates oxygen to the extent of 21.10 per cent of the total gases in milk.
5. The average per cent of oxygen in the total gases found in 116 samples of market milk was 11.24.
6. In order to eliminate any variation of inaccuracies in the test it is imperative that each sample be brought to an oxygen equilibrium. This can be brought about by thoroughly aerating the sample of milk to be tested.

7. Where results for the methylene blue reduction test are to be obtained quickly, it is believed that a uniform addition of potassium iodide or dextrose would maintain the delicacy of the test and also enable results to be obtained much sooner than by following the ordinary procedure.

8. Although no definite statement can be made, it is thought that a 1-20,000 solution of methylene blue will prolong the "lag phase".

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