THE INFLUENCE OF CERTAIN FACTORS ON THE METHYLENE BLUE REDUCTION TEST FOR DETERMINING THE NUMBER OF BACTERIA IN MILK[1]

E. G. HASTINGS, AUDREY DAVENPORT AND W. H. WRIGHT

Department of Agricultural Bacteriology, University of Wisconsin, Madison, Wisconsin

One of the factors, and possibly the most important in determining the quality of milk is its bacterial content, hence, wherever a classification of milk is desired, a bacterial examination is essential. In the dairy manufacturing field, the payment for milk according to its bacterial content has made little headway. The future must bring this change into the industry if the quality of the products is to be maintained at even its present low level, to say nothing of the improvement that should take place. There is no reason why the same price should be paid for high quality milk, as for a milk that is sure to lower the grade of the product for which it is to be used. In the butter industry and in the market milk business certain curative measures can be employed to overcome the negligence of the producer in the production and handling of milk and cream. In the cheese industry, however, prevention, not cure, must be used. A difference in renumeration to the producer is the most powerful lever that can be employed to raise the quality of the milk no matter for what purpose it is intended.

Milk low in bacteria is, generally speaking, of higher quality for cheese making than is milk containing many bacteria. If the cheesemaker seeks to differentiate the milk of his patrons into grades, he must have some means of measuring the bacterial content. Again in the control of market milk the smaller cities have not kept up with the larger in the improvement of their

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milk supplies. They have not been financially able to duplicate the methods of the larger cities. No simple and inexpensive method of determining the bacterial content of milk has been available until recently, and for this reason progress under many conditions has been difficult.

The methylene blue reduction test is the only one that seems to meet the necessary requirements, in so far as simplicity and expense are concerned, for use in many fields, especially in the field of dairy manufacturing. This test is based on the fact that the color imparted to milk by the addition of the dye, methylene blue, disappears more or less quickly, the time required for its disappearance depending on a number of factors, most important of which are the bacterial content of the milk and the temperature at which the test is made. If all controlling factors can be kept constant other than the bacterial content of the milk, the time required for the color to disappear will be determined by the number of bacteria.

The great service which such an inexpensive and simple test might yield to the dairy industries of this country has led the authors to review the literature and to study the test from many points of view. This paper presents simply a summary of our work. In connection with nearly every point considered, many tests have been made. Our conclusions are, therefore, based on much more numerous data than it is possible to present here. It is hoped that this paper may serve to bring the reduction test to the attention of many who may find use for it.

In spite of the widespread use of the reduction test in Scandinavian countries and in other parts of Europe, the method has not been adopted by the dairy industry of this country. Apparently the chief reason for its non-adoption is the belief that it is at best a rough method. The main basis for this conclusion is the fact that the results obtained with it do not correlate exactly with those supplied by the plate culture method. Rahn (1920) has recently emphasized this point and has concluded that the method is not an accurate one. Some of the reasons for the non-correlation of the two methods were given by Hastings (1919). Others will be presented in this paper.

EXPERIMENTAL WORK

The effect of the concentration of methylene blue on the reduction time

In the practical application of the methylene blue reduction test varying concentrations of the dye have been used. Fred (1911) used 1 part of the crystalline dye to 20,000 parts of milk, Jone (1915) 1 part to 50,000; Mueller (1906) 1 part to 40,000; and Kufferath (1919) 1 part to 3000-4000. The latter noted that sterilized milks, by which expression was meant milks heated to the boiling point but not actually sterile, did not reduce the dye. He also noted that raw milk very low in bacteria did not

TABLE 1

Effect of concentration of methylene blue on reduction time

RATIO OF CRYSTALLINE DYE TO MILK	MILK I	MILK II	MILK III	MILK IV
	minutes	*minutes*	*minutes*	*minutes*
1:10,000	25	60	165	
1:20,000	25	45	165	265
1:40,000	22	42	117	240
1:100,000	15	32	95	189
1:200,000	10	27	93	163
1:300,000	8	21	77	163
1:400,000	6		57	160

reduce the dye for several days. Simmons (1919) using a concentration of 1 to 10,000 noted that some milks did not reduce the dye.

It is a well known fact that methylene blue has an antiseptic action. It seems probable that some of the workers who have used the reduction test employed such amounts of the dye that it must have retarded to a marked extent the growth of the bacteria and therefore the reduction time was prolonged.

A considerable number of trials have been made to determine the effect of the dye on the reduction time and the concentration best adapted for employment in the test. Sufficient of the data obtained to illustrate the effect of the varying concentrations on the reduction time of different samples of milk are given in

table 1. It is to be noted from the data that the reduction time is prolonged as the concentration of the dye increases, a fact that can be explained only by the injurious effect of the dye on the activities of the organism.

The tablets prepared and sold by Blauenfeldt and Tvede of Copenhagen are commonly employed in making the reduction test in the Scandinavian countries where it is most widely used. When these tablets are used according to the directions furnished therewith, the color imparted to the milk is practically identical with that obtained when 1 part of the crystalline dye is used to 200,000 parts of milk. Tests made on 24 samples of market milk show that the reduction time is practically the same with the tablets as with 1 part of the crystalline dye to 200,000 parts of milk.

Influence of different brands of dyes

Dyes from various sources, both foreign and domestic, have been compared in different concentrations. The differences in reduction time with any sample of milk, when different dyes are used, are so small as to have no practical significance.

Several writers have stressed the importance of using dyes free from impurities. It seems probable that the impurities which may be present in the grades of dyes that should be used will have little if any effect on the results in the high dilutions in which the dye is employed.

Effect of the age of the methylene blue solution

A number of writers have made the statement that the methylene blue solution to be used in the reduction test should be freshly prepared. Others emphasize the necessity of using sterile solutions of the dye. In order to determine the effect of age on solutions of the dye, tests were made with freshly prepared solutions of dyes from various sources, and with solutions which were two years old. Some of the results are presented in table 2. The results obtained with the fresh solutions and with the old

solutions are practically identical. It is, therefore, believed that the age of the solution will have little if any effect in the practical application of the test.

TABLE 2

The comparative reduction time with fresh and old solutions of methylene blue

	MARTINDALE		HARMER	
	Fresh	Two years old	Fresh	Two years old
	minutes	*minutes*	*minutes*	*minutes*
1:100,000	425	400	146	148
1:200,000	400	370	139	137
1:300,000	380	360	135	137
1:400,000	353	350	121	113

The effect of temperature on reduction time

Most investigators have employed 37° to 38°C. in the reduction test. It is known that some of the milk bacteria, such as the lactic,[2] may grow more rapidly at slightly lower temperatures. It would, therefore, seem that a lower temperature might be more desirable in the practical application of the test. The reducing properties of various pure cultures at different temperatures have been studied by Rahn (1920) and the effect of varying temperatures on the reducing properties of market milk has been studied by Arup (1918) who believes that if milk has been stored at low temperatures, the reduction time at 30°C. will be shorter than at 38°C.

In order to gain some information concerning the reducing power of market milk at different temperatures, a number of tests were made. In each trial approximately 20 samples were employed. If the reduction time of a single sample or the average reduction time of a group of samples at 38°C. is expressed by unity, the reduction time at any other temperature may be expressed by some fraction or multiple of unity. With the great majority of samples the higher temperature has given the most rapid

[2] In this paper the phrase "lactic bacteria" is used to refer to that group of organisms which is primarily concerned in the souring of milk at ordinary temperatures.

reduction. With certain samples of milk the reduction time at 28°C. has been shorter than at 38°C. There seems, however, no reason to deviate from the accustomed temperature (38°C.) in the making of reduction tests. The relative rank of a series of samples is probably of greater importance than the actual reduction time, since it is desirable that the relative quality of milk from a number of sources be determined and improvement of the supply coming from those furnishing the poorer milks be sought. In general, a series of samples of milk will be placed in the same order by the reduction test no matter what the temperature employed in the making of the test. The temperature at which the average milk will reduce most quickly is, therefore, the best one to be used.

TABLE 3

The influence of temperature on the relative average reducing power of a series of samples of milk

TRIAL	38°C.	33°C.	28°C.
1	1	1.4	2.0
2	1	1.5	1.9
3	1	1.7	2.1
4	1	1.5	2.0
5	1	1.6	1.9
6	1	1.4	2.0

Are there non-reducing bacteria?

Previous investigations claim to have found that certain bacteria would not reduce methylene blue in milk. It seems to the writers that these observations are in error. An organism which grows slowly in milk, either because of temperature, nutritive conditions, or an excessive amount of methylene blue will not be able to use oxygen as fast as it can diffuse into the milk. Such an organism would be classed as a non-reducer. Under appropriate conditions such an organism will reduce the dye. In a mixture of organisms it will exert an effect. There is every reason to believe that every organism growing in the milk assists in the reduction of the dye. All do not function in the same degree, however.

The relation of the living cell to reduction

The effect of heat on the reduction time is evidence that the cell itself and not its enzymes are the causal agents in the reduction of the dye. The data presented in table 4 show that temperatures that are not supposed to affect enzymes influence the reducing action of milk. The increase in reduction time with the increase in temperature may indicate the varying resistance of different cells of the same organism or a complexity of bacterial flora in the milk, or possibly it may indicate both conditions. The

TABLE 4

The effect of heating milk to varying temperatures on its reducing power

TEMPERATURE TO WHICH HEATED	TRIAL 1	TRIAL 2
°C.	*minutes*	*minutes*
40	17	45
45	17	50
47	17	50
50	17	55
52	32	77
54	42	107
56	57	135
58	139	155
60	189	205
62	242	240
64	377	305

effect of such a weak antiseptic as boric acid in concentration of 1 part per 2500 parts of milk approximately doubles the reduction time. The effect of increasing amounts of methylene blue in lengthening the reduction time is to be traced undoubtedly to the antiseptic action of the dye. The influence of these factors, heat and antiseptics, finds its explanation in their influence on the living cells, and it is evidence that the reduction of methylene blue is very intimately connected with the vital processes of the cell rather than with any extracellular by-products.

Effect of reaction of the milk on reduction time

Mueller (1906) studied the effect of varying reactions on the reduction time and also studied the effect of adding sodium carbonate and sodium bicarbonate to sour milk. His conclusion from rather extended observations was that the reduction time of milk in which a considerable amount of acid has been developed is not changed by neutralization of the acidity. Orla-Jensen also made similar observations. This is exactly what one would expect for it is well known that the rapidity of growth of bacteria in milk does not decrease until the acidity has reached a point at which the milk will curdle at ordinary temperatures. In other words, the reaction of any sample of milk to which the reduction test would ever be applied in practice would not be such as to interfere with the test.

Effect of shaking on the reduction time

It is recognized that one of the reasons why the plate culture does not reveal the exact number of living cells in the milk is the occurrence of clumps of organisms therein. The ordinary lactic bacteria tend to occur in twos, other forms may occur in much larger aggregates. Each aggregate, of course, gives rise to a single colony. This has led the committee of the American Public Health Association that formulated standard methods for the bacteriological examination of milk to suggest that the plate culture count of milk be spoken of as such rather than to speak of the number of bacteria per cubic centimeter. This source of error in the plate culture method should not be present in the reduction test since undoubtedly each cell of such clumps as are likely to occur in milk will function as though the other cells were not present. This should indicate that shaking would have no effect upon the reduction time. Dons (1914) found this to be true. Orla-Jensen confirmed this observation. In our own work some attention has been paid to this point. We have found that the agitation of the milk has practically no effect on its reduction time.

Relationships existing between different groups of bacteria in milk

Efforts have been made by numerous investigators to correlate the results of the plate culture method with those obtained with the methylene blue reduction test. When the correlation was not an exact one, it has had the tendency to minimize the value of the reduction test. Rahn (1920), in a recent article, has again raised this objection and considers the reduction test only an approximate method and not comparable in value to other tests.

It seems apparent that these investigators have not given adequate consideration to the relationships which must exist between the various groups of bacteria that are present in milk. Two kinds of organisms may have a favorable reaction upon each other; thus the growth of both may be stimulated. Again, one kind may be favored by the previous growth of another, or the development of one group may exert an inhibiting action upon another. An important example of this is the influence of the acid-forming group upon the liquefying bacteria. Again, inhibition of both organisms may result. When one considers that there are many groups of organisms present in milk, it is evident that the relationships existing between them must be most complex. In the plate method these relationships are avoided when the plates are not too thickly seeded. It is well known to every bacteriologist that thickly seeded plates give no correct idea of the quantitative or qualitative bacterial content of the milk. Plates heavily seeded may give the impression that nothing but lactic bacteria are present in the milk, while more thinly seeded plates from the same sample may reveal many other kinds of bacteria. The existence of these relationships between different groups of bacteria in milk and their absence in plate cultures is undoubtedly an important cause for a lack of correlation between the results obtained with the plate culture method and with the reduction test.

From many points of view the bacteriologist is chiefly interested in those organisms which are actually growing in milk. It seems probable that the methylene blue reduction test because it is influenced by these relationships measures more accurately than does any other method the bacterial activity in the milk.

Variation in reducing action of different kinds of bacteria

Another cause for the non-correlation of results obtained by the plate culture method and by the reduction test is the variation in the reducing action of different milk bacteria. Efforts have been made by a number of investigators to compare organisms with reference to their reducing power. Orla-Jensen (1912), Barthel (1908), Weigmann and Wolff (1916) and Rahn (1920) have worked along this line. The conclusions which they reached in regard to the reducing action of the different milk bacteria were quite dissimilar. Orla-Jensen and Barthel asserted that the ordinary lactic bacteria were not to be classed as strong reducing organisms, while Rahn believed them to be the most active in the reduction test. The methods used by these investigators varied. Orla-Jensen and Barthel added methylene blue to the cultures that were already fully developed, while Rahn inoculated the milk with the culture and then added the methylene blue. In the latter instance the conditions would be exactly similar to those present in the practical application of the test; namely, the organisms would be actively growing during the time of the test while, in the method used by Orla-Jensen and Barthel, only the dormant organisms would be present.

In our own work many efforts have been made to obtain some idea of the relative reducing action of different bacteria. Various methods have been employed. The one which gave the most satisfactory results was as follows: A series of tubes of fresh milk which had been heated to the boiling point were inoculated with a constantly decreasing amount of an active lactic culture. The same tubes were then treated in the reverse order with the culture of the organism to be compared with the lactic. Each tube of the series, therefore, contained the same volume of the mixed cultures of the two organisms, but the relation of the two cultures to each other was different in each tube. If the organism to be compared with the lactic had the same reducing action, and if the organisms did not influence each other unfavorably and grew at approximately the same rate, the time for reduction should be much the same in the different tubes of the series. This con-

clusion is based on the supposition that the cultures of the various organisms contained approximately the same number of cells. Although such a condition probably did not exist nevertheless the variation in number should not influence the results to such an extent as to make them of no value. Some of the results obtained are given in table 5.

It is to be noted that with each organism tested the reduction time has increased as the inoculum with lactic decreased. If the compared organism had reducing powers equal to the lactic

TABLE 5

The reducing action of different bacteria in milk

INOCULUM			COMPARED ORGANISMS												
Tube	Lactic bacteria	Compared organism	1	2	3	4	5	6	7	8	9	10	11	12	13
	cc.	*cc.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*m n.*	*min.*	*min.*	*min.*	*min.*
1	1.9	0.0	8	10	14	12	13	12	31	15	15	15	15	8	8
2	1.7	0.2	11	12	16	14	15	13	37	15	15	15	15	11	8
3	1.4	0.5	16	22	20	18	15	19	43	20	20	16	20	16	16
4	1.1	0.8	21	26	30	20	19	25	64	24	24	24	24	22	22
5	0.8	1.1	29	35	30	28	30	38	71	33	33	33	33	35	35
6	0.5	1.4	43	62	56	50	45	58	81	44	45	45	80	70	70
7	0.2	1.7	80	87	71	57	52	106	98	67	56	56	67	120	120
8	0.0	1.9	140	173	93	95	85	178	335		107	260	260	360	185

Organisms 1 to 6 inclusive were isolated from milk. They represented the udder flora. Organisms 7, 8, and 9 were representatives of the aerogenes group; 10 and 11 of the colon group; 12 and 13 of the Bulgaricus group.

bacteria this increase in reduction time would not have occurred for the increasing amount of the compared organism would have balanced the decrease in lactics. No organism tested was at all comparable to the lactic in reducing power. The great difference in reduction time between tubes 7 and 8 is evidence of the marked reducing action of the lactic bacteria in comparison with the other bacteria. Tube 7 contained in each trial a small amount of lactic bacteria, tube 8 none.

The reducing action is, of course, influenced by the rate of growth of the various organisms. The temperature used in the

test, namely, 38°C., is more favorable for the organisms of the colon-aerogenes group than for those of lactic group. In spite of this handicap the lactic organism exhibits much greater reducing action than do the members of the colon group. This observation agrees with what is known concerning the reducing action of these organisms in litmus milk.

The relation of reduction time to keeping quality

The desirability of a laboratory test that would give some evidence of the keeping quality of milk would be of value in grading it. In order to collect some information as to keeping quality as measured by the development of acid, and the reduction time of the milk, a number of tests were made. In the first trial tubes of fresh milk, very low in bacteria, were inoculated with sour milk in such a way that the number of bacteria added would decrease from tube to tube in any one series. The milk was stored at approximately 8°C. and after ninety-six hours the acidity of the milks was determined. The results of two such trials are presented in table 6.

It is to be noted from table 6 that the correlation between the reduction time and the development of acidity was perfect. It is to be remembered that lactic bacteria predominated in the milk used in these trials. In other words, the bacterial flora was much less complex than that of ordinary market milk.

In samples of ordinary milk not only acid-forming, but also non-acid-forming and alkali-forming bacteria will be present. There would be wide variations in rapidity of growth of the different kinds at varying temperatures. Such complex relations would probably destroy to a considerable extent the correlation between acid development and reduction time. In order to collect some information concerning this point, acid and reduction tests were run on samples of market milk when fresh, and also after storage for twenty-four and forty-eight hours, at approximately 10°C. In table 7 the samples were ranked according to their reduction time when fresh, the sample reducing most rapidly being placed first.

It is to be noted that the acidity has developed more rapidly in the samples of milk which showed a short reduction time when fresh, and in a general way there is a correlation between reduc-

TABLE 6

The relation of the keeping properties of milk to reduction time

SAMPLE	TRIAL I	
	Reduction time	Acidity after ninety-six hours at 8°C.
	minutes	
1	150	0.57
2	186	0.56
3	217	0.51
4	258	0.44
5	303	0.33
6	340	0.28
7	355	0.24
8	371	0.22
9	403	0.20
10	411	0.19

TABLE 7

The relation of acidity and reduction time in milks after varying periods of storage

SAMPLE	ACIDITY AND REDUCTION TIME AFTER 0 HOURS AT 10°C.		ACIDITY AND REDUCTION TIME AFTER 24 HOURS AT 10°C.		ACIDITY AND REDUCTION TIME AFTER 48 HOURS AT 10°C.	
	per cent	*minutes*	*per cent*	*minutes*	*per cent*	*minutes*
56	0.222	125	0.261	10	0.344	1½
30	0.166	205	0.205	11	0.355	2
369	0.188	305	0.194	55	0.210	7
59	0.188	340	0.194	265	0.205	11
86	0.188	360	0.200	260	0.200	22
130	0.210	360	0.210	295	0.216	22
354	0.216	400	0.228	49	0.235	5
135	0.166	400	0.183	335	0.177	160
356	0.188	475	0.188	335	0.177	75
359	0.194	503	0.188	355	0.177	90
357	0.194	510	0.200	385	0.222	15

tion time and keeping quality if the latter is to be measured by acid production. There are, however, certain deviations from this correlation; for example, sample number 135 has shown practically no increase in acid during the storage period, while

the reduction time has decreased. The same is true of samples 356 and 359. In other words, in these samples the effect of acid-forming bacteria if they were present, has been overbalanced by alkali-forming organisms. The latter would, of course, exert an effect on the reduction time. They would not, however, influence the keeping quality as measured by acid development.

It seems from the results obtained that the reduction test will reflect only in a very general way the keeping quality of a sample of milk. It is undoubtedly true that the other methods for the examination of milk, such as the plate culture method and the direct microscopic examination indicate quite as well as the reduction test, the keeping quality. On account of the great complexity of bacterial flora in milk, it seems impossible for the keeping quality to be determined by any one test, or probably by any group of tests.

Directions for making the reduction test

A stock solution of medicinal methylene blue or that recommended for use in staining bacteria is prepared by dissolving one part of the crystalline dye in 2000 parts of water. A portion of this solution is further diluted shortly before use until the concentration is one of the dye to 20,000 of water. One cubic centimeter of this solution in 10 cc. of milk gives a dilution of slightly over 200,000. The solution should not be filtered since this will remove a considerable portion of the dye, especially is this true with dilute solutions.

Tubes of approximately the same diameter and of such a size that 11 cc. will not fill them over one-half full are used. The samples should be collected with the same care as for any bacteriological examination. The tubes should be clean and steamed or boiled shortly before using. It is unnecessary to sterilize them. The 10-cc. sampling pipette should be clean and should be rinsed at least three times with boiled water between samples. After the addition of the dye the contents of the tube should be mixed by closing the tube with the thumb or palm and inverting once or twice. Wiping the moistened area of the hand with a clean

towel will be sufficient prevention of contamination from sample to sample.

A water bath which can be kept at 38°C. in which the tubes can be placed is the only apparatus needed, other than the tubes and pipettes.

The frequency of observation will be determined by the number of grades into which one wishes to divide the milks. There will be little use under ordinary conditions of extending the period of observation over six hours.

Reading the test

It is usually thought that the distribution of bacteria in a recently mixed sample of milk is uniform, in other words, that any small quantity will be an exact duplicate of another sample. There are a number of reasons for believing that this is not true: especially in milk of low bacterial content, among them is the appearance of some of the milk in the reduction test. In the great majority of cases, the color will disappear uniformly from all parts of the milk. In other cases the color may disappear in an uneven way. A small zone or area may lose the color before there is any apparent reduction in other zones. In still other cases the color may persist in the lower layers of the tube after the upper part is completely decolorized. The more common occurrence is the persistence of the color at the top. Vitoux (1920) in his description of the reduction method as used in Holland states that the upper one-fourth of the tube should be disregarded in reading the test. Another method, less convenient in practice, is to take as the end point, the time when no blue color is to be noted after the milk has been mixed. The slight amount of unreduced dye in the surface layer will not be noticeable when mixed with the entire quantity of milk. Again, the color may be reduced to such a point that the milk appears white except when compared with a control tube of milk when a distinct bluish tinge will be noted. This residue of color may persist unchanged for a considerable period. We have not been able to relate this to any factor. It is probable that it is of small importance.

SUMMARY

The importance of the bacterial content of milk in determining its quality is discussed and the need of some simple and inexpensive method for the bacteriological analysis of milk is presented. The methylene blue reduction test seems to meet the need.

The literature treating of the reduction test has been reviewed and a study has been made of a number of factors which influence the results obtained with the test. In the work herein reported attention has been directed to the influence of the concentration of the dye, to the age of the solution of the dye, and to the source or brand of the methylene blue.

The influence of temperature on the reduction time has been investigated.

The direct dependence of the test on the vital activities of the cells is shown by the effect of heating the milk to relatively low temperatures and by the effect of antiseptics.

The relationships which may exist between different groups of bacteria are discussed and data are presented to illustrate the effect of these relationships in influencing the reduction time.

The reducing action of different groups of milk bacteria has been studied as well as the relation of reduction time to keeping quality.

Directions for making the test are given.

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