STUDIES ON OXIDATION-REDUCTION IN MILK

I. OXIDATION-REDUCTION POTENTIALS AND THE MECHANISM OF REDUCTION[1]

H. R. THORNTON AND E. G. HASTINGS

Department of Agricultural Bacteriology, University of Wisconsin

Received for publication June 20, 1929

The recent work of Clark and his co-workers (1928, I–X) makes desirable a reinterpretation of the meaning of dye reduction in milk, and provides a new and excellent avenue of approach to the study of oxidation-reduction phenomena in this biological fluid.

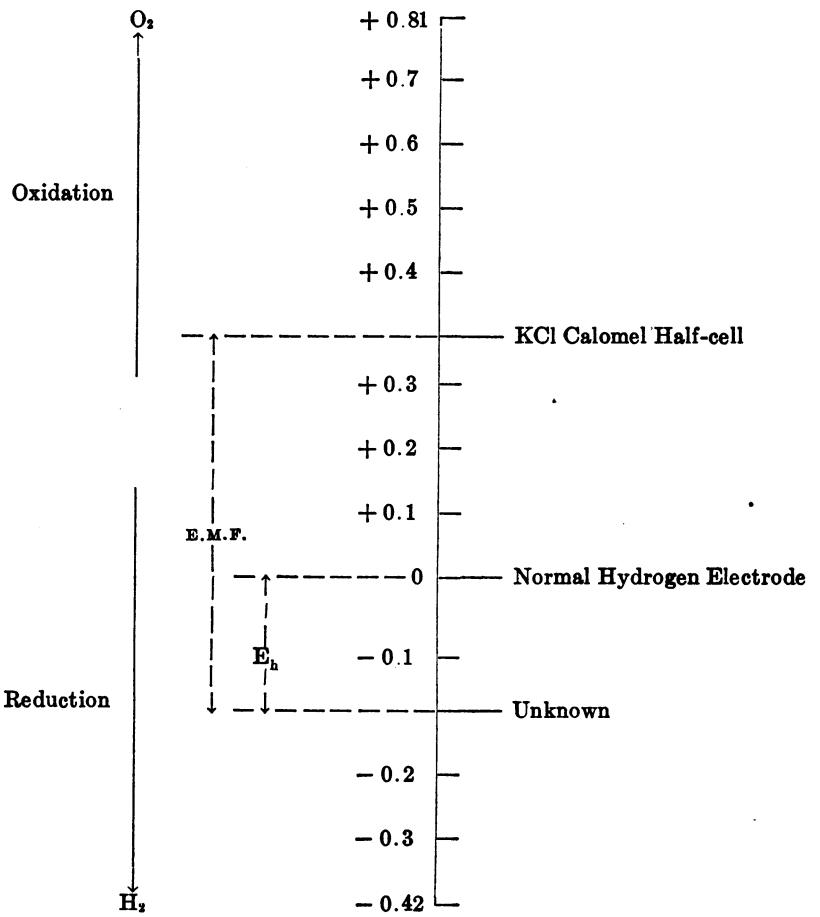
Oxidation is defined as the process in which a substance takes up positive, or parts with negative, charges, while reduction is the process in which a substance takes up negative or parts with positive charges. These electronic changes may be followed in milk potentiometrically. Clark (1925), Thornton and Hastings (1927, 1928), and Thornton (1927, 1929) have reported such experiments. The present paper is a fuller presentation of the work reported earlier by the latter two authors.

OXIDATION-REDUCTION POTENTIALS

If an electrode of one of the regal metals is immersed in milk and connection is made to a potassium chloride calomel half-cell through a potentiometer and the circuit completed through a potassium chloride agar bridge, the potential difference between the milk and the calomel half-cell is easily measured. By a simple computation this potential may be referred to a standard zero, the normal hydrogen electrode. The difference in potential between the normal hydrogen electrode, the potential of which is

[1] Published with the permission of the Director of the Agricultural Experiment Station.

arbitrarily assumed to be zero, and the milk is termed E_h. A schematic representation of these relationships is given below. In the illustration chosen the observed E.M.F. was 0.4864 volt, the unknown being negative to the calomel cell. The E_h is, therefore, -0.15 volt.



For the work reported in this paper a constant temperature bath operated at 37.5°C. ±1° was fitted with hydrogen leads to accommodate 6 samples. Therefore the pH values of all of our

samples were determined at frequent intervals. Wires from 6 electrodes led through a 6-way switch to a Leeds and Northrup Type K potentiometer. Platinized-platinum electrodes were used in hydrion determinations and gold-plated platinum electrodes for oxidation-reduction potential determinations. A 0.1 N potassium chloride calomel half-cell was used. Connection was made through a saturated potassium chloride liquid junction

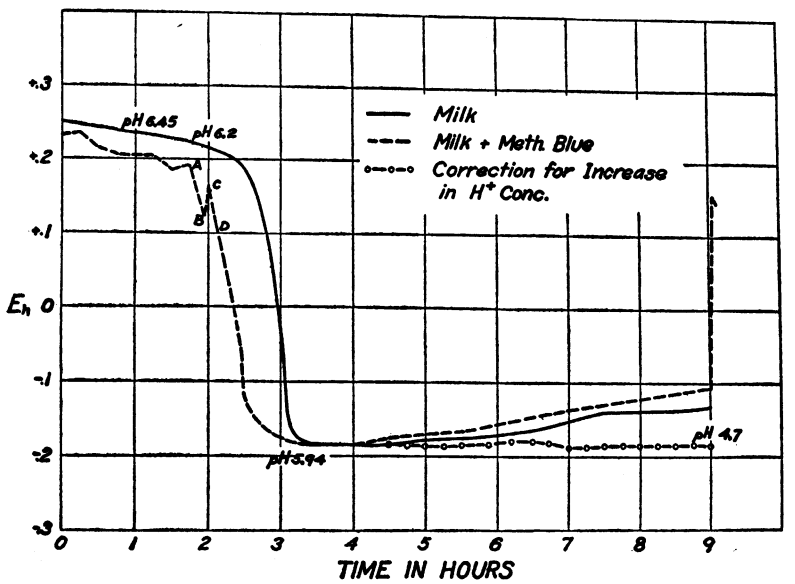


FIG. 1. POTENTIAL-TIME CURVES OF A SAMPLE OF MARKET MILK WITH AND WITHOUT THE ADDITION OF METHYLENE BLUE

by means of saturated potassium chloride agar bridges. All readings of E.M.F. are reported in volts in terms of E_h.

The oxidation-reduction potentials of a number of milk samples were followed at few minute intervals. The results of one such experiment are given graphically in figure 1. The solid line represents the potential changes in the milk alone. In the case of the broken line the standard amount of methylene blue (1 part of dye to 200,000 parts of milk) had been added. This latter milk was shaken at half-hour intervals as gently as possible to

incorporate only the minimum amount of oxygen and at the same time prevent the rising of the butter-fat. These curves show distinctly the effect of shaking upon the reduction potential and time. This effect will be given full consideration in a later paper. At point *A* no reduction of the methylene blue could be observed, while at point *B* the milk had turned white. The vessel was then shaken and the blue color reappeared. The potential was then read as at *C*. Complete visual reduction had again taken place at *D*. Shaking was then discontinued till the ninth hour. This last shaking caused the potential immediately to swing almost to the positive extreme, and a faint blue color reappeared in the milk. A further reading within five minutes showed the potential to be changing rapidly toward the negative side. When the changes in the potentials as read with the hydrogen electrode were subtracted from the potential values as read with the gold-plated electrode and the values plotted, the circle-line curve was obtained. This suggests that the upward trend of the curve in the last five hours of the experiment was due to the increase in the hydrion concentration.

Examination of this figure shows that the dye decolorized in a zone about 0.1 volt more positive than the theoretical for methylene blue at this pH (see Clark, 1925). This seems to indicate salt effect or the influence of another oxidation-reduction system or systems. This effect is not constant but varies in different milks, and we wish to point out that, at present, caution should be used in interpreting reduction intensities in organic complexes in terms of potential on the basis of dye reduction. We have observed complete visual reduction of methylene blue in different milks at E_h values as low as +0.075 volt (rH 14.5) and as high as +0.225 volt (rH 19). rH is defined as the logarithm of the reciprocal of the hydrogen pressure.

Figure 2 represents a similar experiment except that the milk containing the dye was left undisturbed until after decolorization had taken place. The sharp inflection in the solid line was caused by the changing of electrodes at this point. Reduction of the dye had taken place at *A*. The potential change was so rapid and the end-point of reduction so indefinite that the

points of reduction are, perhaps, only approximate. At *B* the milk containing the methylene blue was shaken with the result seen in the graph. The blue color returned to the milk and this had again disappeared when *C* was reached.

The positive E_h limits of all the milks examined lay between +0.2 and +0.3 volt. This corresponds closely to the limits reported by Clark and others for milk and some other biological fluids. The negative limits reached by all our milks were re-

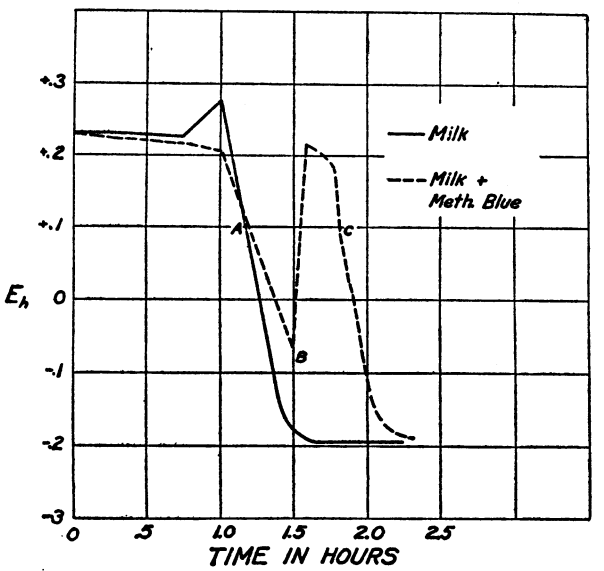


FIG. 2. POTENTIAL-TIME CURVES OF A SAMPLE OF MARKET MILK WITH AND WITHOUT THE ADDITION OF METHYLENE BLUE

markably uniform, approximating E_h −0.2 volt. This is the limit of reduction potential reached by a pure culture of *Streptococcus lactis* growing in milk as reported by Clark (1926), and suggests that the predominating influence in our milks was that of the lactic bacteria. Very little work, however, has been done on the oxidation-reduction potentials of pure cultures of bacteria, so that a definite conclusion cannot be drawn.

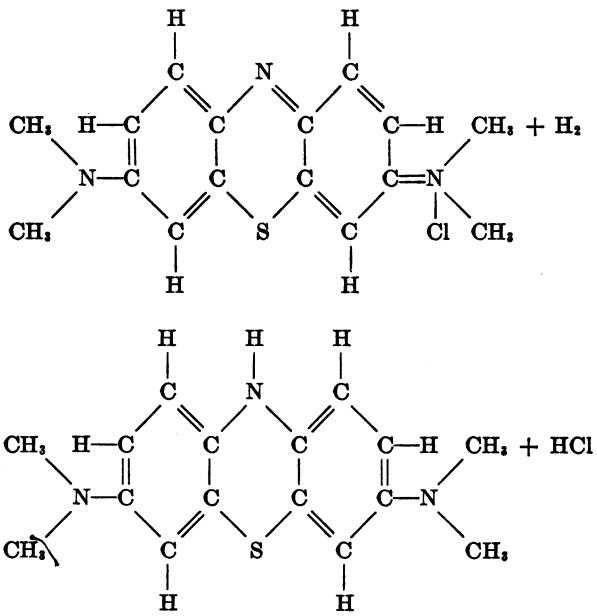
"A solution may be said to be *poised* when it tends to resist change in E_h on addition of an oxidizing or reducing agent."

These curves reveal a slight *poising* effect of the methylene blue in milk. This effect is so small as to be almost negligible when the standard dye concentration is used.

THE MECHANISM OF REDUCTION

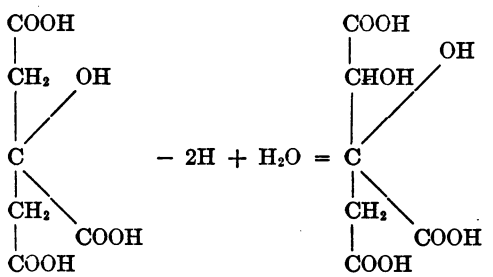
The rôle of hydrogen

The accepted equation for the reduction of methylene blue to methylene white is given below. It will be seen from this equation that the reduction of this dye involves no change of oxygen, since there is no oxygen in the methylene blue molecule. Two atoms of hydrogen are concerned, however. One atom withdraws the chlorine from the basic terminal nitrogen. A shifting of the double bonds takes place leaving the bridging nitrogen unsatisfied. The other atom of hydrogen links up with this nitrogen forming methylene white.



This immediately suggests the interesting problem of the source of the hydrogen involved which has been a matter of

controversy for years. Lactose, which is present in milk to the extent of about 5 per cent, is a reducing sugar and must receive consideration as a contributing agent in the reduction of dyes in milk. Of recent years attention has been focused upon a group of compounds which have been called "metabolites." Hopkins (1921) believes glutathione to be the hydrogen donator and acceptor in oxidation-reduction processes in animal tissues. He states that glutathione is a compound of cysteine and glutamic acid with a free S—H group. Harding and Cary (1926) have demonstrated this compound in the blood of the cow in larger quantities on entering than on leaving the udder. Viale (1925) and others have shown that other such compounds having the sulfhydryl group enhance the reducing powers of milk. Thunberg (1925) found that succinates effect reduction potentials. The power of succinates and citrates to aid in reduction in milk has been demonstrated by Barthel (1925). He believes the citrate in the milk to be the hydrogen donator according to the following equation:



This reaction, he thinks, is catalyzed by the milk salts. His experiments showed increasing acceleration of the reduction of methylene blue in milk on the addition of increasing amounts of sodium citrate or succinate. Quastel (1926)

examined 103 substances as possible donators or acceptors of hydrogen (using the methylene blue technique) in the presence of bacteria (*B. coli*) and of these 56 are activated. That is, of the 56 substances some reduce methylene blue, while others oxidize leucomethylene blue under conditions (pH 7.4 and 45°) when they are apparently quite inactive in the absence of the organism.

Clark (1926) published potential-time curves showing the effect of the addition of such metabolites as sodium succinate and glutathione upon the potentials of suspensions of washed yeast cells.

To study the effect of the addition of cysteine upon the reduction of methylene blue in milk two tubes of the same milk containing methylene blue were incubated at 37.5°C. for thirty minutes at which time cysteine was added to one tube. The

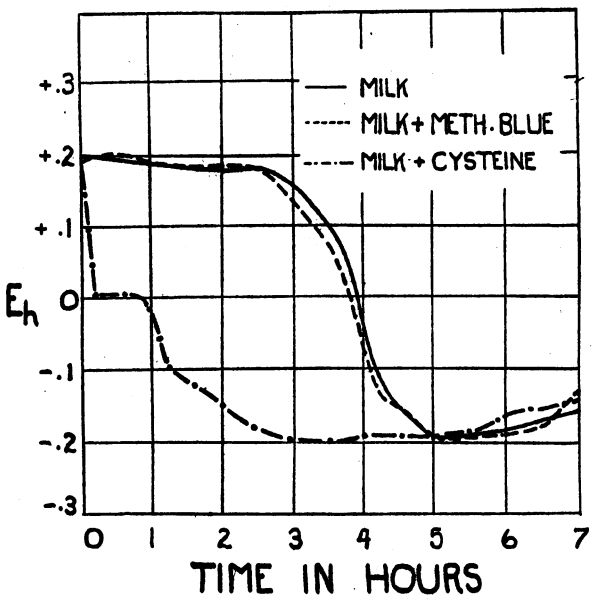


FIG. 3. POTENTIAL-TIME CURVES OF A SAMPLE OF MARKET MILK WITH AND WITHOUT THE ADDITION OF METHYLENE BLUE AND OF CYSTEINE

methylene blue in this tube decolorized in another hour, or in one and one-half hours from the start of incubation. The methylene blue in the tube containing no cysteine reduced in nine hours from the start of incubation. Figure 3 shows the effect of the addition of cysteine in a concentration of 1 part cysteine to 1000 parts milk upon the potentials in a sample of milk. Attention is called to the uniformity of the negative limits of potential. If the limit is due to the influence of *Streptococcus lactis*, then

this influence is greater than that of cysteine in this concentration. Table 1 shows the results of another experiment in which a number of substances were added to duplicate tubes of milk and methylene blue reduction times noted.

The question, therefore, of the hydrogen source in the reducing processes in milk is not settled as yet. It seems probable that a number of the constituents of the milk are concerned. For the practical application of the methylene blue reduction test for quality in milk this is of no great importance for, as will be shown later, the inherent reducing "capacity" of milk is not over-taxed by the concentration of dye used in this test.

TABLE 1

The effect of the addition of a number of substances upon the reduction time of methylene blue in milk

SUBSTANCE	CONCENTRATION	REDUCTION TIME
Sodium citrate	1:1000	7:30
Sodium citrate	1:500	7:30
Sodium nitrite	1:1000	9:30
Sodium nitrate	1:1000	7:30
Cysteine	1:1000	2:00
Control		7:45

Note: Reduction times are reported in hours and minutes. Thus 7:30 means 7 hours and 30 minutes.

The rôle of oxygen

It is a common observation that the blue color returns to reduced methylene blue milk mixtures on shaking with air. That this is due to the oxygen in the air is proved by the fact that neither hydrogen, nitrogen, nor carbon dioxide will cause the oxidation of methylene white to methylene blue in milk, while oxygen will do so.

This oxygen relationship may be demonstrated in a number of ways. On heating an alkaline solution of a reducing sugar, to which methylene blue has been added, reduction takes place. On cooling and shaking with air, the blue color returns. This may be repeated many times. If the unheated solution is left exposed

to the air in a thin layer, reduction does not take place. If, however, diffusion of atmospheric oxygen into the liquid is prevented by a paraffin or vaseline seal, reduction will take place at room temperature. The dye is also reduced in such a solution in an uncovered test-tube where the surface exposed to the air is small in proportion to the volume of the liquid. Any of these reduced solutions of sugar will regain their blue color on shaking with air. Thus the sugar solution consumes oxygen and that oxygen bears an important relationship to reduction.

If a sterile methylene blue milk mixture is allowed to stand in a thin layer with the surface exposed to the air, reduction does not take place. If the same mixture is placed in test-tubes and sealed with vaseline, or even with the normal cream layer of

TABLE 2

The effect of exhaustion upon the reduction time of methylene blue in milk

EXHAUSTION TIME	REDUCTION TIME
minutes	
0	4:05
2	2:50
15	1:15
30	0:45

whole milk, and incubated at room temperature, reduction ultimately takes place. If a sterile mixture of methylene blue and skim milk is placed in test-tubes and the test-tubes sealed off in a flame, reduction of the dye will take place. The reduction period may be three or even six months depending upon the ratio of the volume of milk and of air sealed above the milk. That the milk has consumed the oxygen, thus allowing reduction, is shown by the quick return of the blue color if the tubes are opened and the contents shaken with air.

In one experiment, the reduction time of methylene blue in a sample of milk was found to be forty-five minutes. The reduction time in a duplicate sample through which oxygen had been bubbled for a few moments was one hour and forty-five minutes. Harvey (1919) has shown that the reduction times

in the Schardinger reaction vary with the oxygen content. We have found the same to be true in the reduction test as is shown in table 2. For this experiment quantities of the same milk were exhausted by means of a waterpump and the reduction times noted.

Barthel's experiments with deaerated raw and heated milks and the work reported later in this paper with similar milks prove without question that oxygen is an important factor in dye reduction by milk. Scrutiny of figure 1 of this paper will show the effect of oxygen upon the reduction potentials in milk and that these potentials depend, in the main at least, upon the oxygen content of the milk.

In his series of papers on oxidation-reduction Clark reports that certain of the indolphenols will reduce immediately in milk. Drs. Clark and Cohen have very kindly supplied us with a number of their dyes and we have confirmed these observations with two of the indophenol indicators. These dyes were reduced almost immediately, even in fresh milk of low bacterial content. The ranges of reduction intensity within which these indicators pass from the oxidized to the reduced state lie outside the range of reduction intensity in milk toward the positive end of the E_h scale. Clark reports reduction of 2-6 dichlor-indophenol by a suspension of washed yeast cells "while a vigorous stream of air was being passed through the suspension." This suspension was unable to reduce methylene blue appreciably under anaerobic conditions.

Other dyes, reducing over a potential range more negative than that of methylene blue, will be reduced in milk in greater time than that necessary for the reduction of methylene blue and reduction will usually be in the order in which these dyes fall upon the E_h scale.

We have found that when the potential has reached the negative limit minute amounts of oxygen will cause the potential to swing rapidly and markedly toward the positive side (fig. 1). This sensitivity toward oxygen appears to increase with the fall in potential. If, now, we assume that the bacteria are reproducing every half hour, then the growth of the bacteria within

any given half hour represents one-half of the total oxygen-consuming power of the bacteria up to the end of that time. It is improbable that this assumption is ever correct. In one instance, however, plate counts made every half hour roughly doubled in that period of time. The main drop in potential from the positive to the negative side usually takes place in about one half hour. Therefore, the oxygen-consuming power of the bacteria during this period must be relatively great. This has led one of us (Thornton, 1927) to conclude that, while small amounts of oxygen are probably present at the time of decolorization of methylene blue, anaerobic conditions obtain when the lower limit of potential is reached. This opinion we now wish to revise. It seems more satisfactory to suppose that anaerobic conditions are not reached at the negative potential limit as shown in figures 1, 2 and 3, but that an equilibrium is established at this point and the oxygen content of the milk remains constant. We are not in a position to give an opinion as to the influence of oxygen upon the potential of aqueous solutions which have reached a reduction intensity more negative than those reported in this paper. Clark (1924) states

> In a case where we allowed bacterial reduction of indigo carmine to proceed to 80 per cent reduction we measured the potential electrometrically and calculated therefrom the oxygen pressure. It came out 10^{-36} atmospheric. Using the data of Millikan on the number of molecules per gram mole of gas we calculate that less than one discrete individual molecule of oxygen was present at equilibrium in 10^{13} liters of the culture.

It seems apparent, therefore, that the reduction of methylene blue by a culture is not necessarily an indication of anaerobic conditions, but merely that a certain partial pressure of oxygen has been reached. This possibly explains some of the unsatisfactory results bacteriologists have experienced when using this dye as an indicator for oxygen in anaerobic culture work. It is probable that the effect of oxygen upon the reduction intensities of different milks and different media is not quantitatively identical.

The theory of Wieland (1922) that the dissolved oxygen be-

comes the acceptor for the hydrogen is the popular explanation today for the disappearance of oxygen in biological reductions. According to this theory hydrogen combines with the oxygen to form first H_2O_2 and then H_2O as represented by the following equations:

$$O{=}O + H_2 = HO{-}OH$$

$$\begin{array}{ccc} OH & & H \\ | & + & \\ OH & & H \end{array} \quad = \quad OH_2 \quad OH_2$$

The rôle of enzymes

The early workers believed the phenomenon of methylene blue reduction in milk to be due to enzyme action, hence the name "Reductase Test." The reductase was supposed to be elaborated by the growing bacteria. This is well illustrated by the following conclusion of Fred (1912):

> Reductases are formed by the growth of microörganisms and do not occur in milk when first drawn. The reduction of methylene blue, free of formalin, is very complex and is no doubt aided by the changes of matter during assimilation. Very probably both intracellular and extracellular products take part in the reduction.

Most workers have failed to recognize the relationship of oxygen to the reduction of dyes in milk and in their experiments provided no substitute for the oxygen-consumption of the bacteria when these were not present or were present only in small numbers. The enzyme theory of reduction has not been a satisfactory one and leaves unexplained such phenomena as the reduction of dyes in sterile milk and other bacteriological media.

Burri and Kürsteiner (1912) reported reduction in fresh milk of low bacterial content in which bacterial growth was inhibited by antiseptics. They concluded that this reducing power is inherent in the milk and is of non-bacterial origin. Barthel (1917) observed reduction of methylene blue in freshly autoclaved milk within 1 hour and 50 minutes. This precludes entirely a bacterial enzyme theory. There remains, however, the possibility that the reducing power of the milk is created or increased be-

cause of chemical changes of the milk constituents during the heating process. The creation of a reducing property in milk by heating seems out of the question in the face of further experiments by Barthel in which he removed the oxygen from fresh raw milk of low bacterial content with hydrogen, carbon dioxide, and nitrogen and obtained reduction, in one case in thirty minutes.

With a similar use of molecular hydrogen or carbon dioxide we have observed reduction in thirty minutes at 37.5°C. in fresh raw milk of low bacterial content. Using the same technique on a freshly autoclaved nonaerated milk (except for the aeration consequent upon the careful addition of sterile but aerated methylene blue solution) reduction was complete in ten minutes. In our experiments this reduction time includes that time necessary to wash out the residual oxygen as well as the time taken for the actual reduction of the dye. We used 10 cc. of milk while Barthel used 40 cc. Apparently an inherent reducing power is present in milk at the time it is drawn from the udder. The heat of autoclaving removes sufficient oxygen to allow the potential to pass through the range of methylene blue. Diffusion of atmospheric oxygen into the milk on cooling causes this potential to swing to the positive side again and the leucobase undergoes oxidation. The assumption that heating increases the reducing power of milk is, therefore, not necessary in order to explain the reduction of dye in these experiments.

It is our opinion that failure to recognize the reducing properties of various media in which bacteria or other cells are suspended and insistence on attributing reduction solely to the suspended cells are instrumental in masking much that would aid in explaining biological reductions. Clark (1926) in reporting on reduction potentials in cell suspensions says

> It is well known from the work of Battelli and Stern, Thunberg, Harden and Morris, and others that the ability of a suspension of cells to reduce methylene blue is almost completely removed by exhaustive washing with water. We have followed the potentials developed when washed cells (yeasts, muscle and liver) are suspended in a deaerated buffer in the electrode vessel already referred to. The more exhaustive the

washing, the less definite become the potentials, until, for example, suspensions of six times washed yeast or washed muscle hold the electrode only most erratically.

It is likely that all organic complexes consume oxygen. It seems highly probable that any medium, in, or on, which bacteria or yeast can grow, will also possess reducing powers. Small amounts of this medium will be transferred to the buffer solution in which the cells are suspended and the quantity will diminish with repeated washings. Among the cell constituents there are, without doubt, compounds not entirely dissimilar to those making up the common laboratory media. There is a possibility that some of these diffuse into the surrounding buffer solution increasing the reducing properties of that solution. It is probable that repeated washing would lessen these water-soluble and diffusable constituents. If this is so, then the problem of biological oxidation is, to some extent at least, simplified.

Every bacteriologist has noticed that litmus milk is reduced as it comes out of the autoclave and quickly becomes colored again as it cools. If oxygen diffusion is prevented by a seal of vaseline or butterfat, a slight purpling will take place coincident with cooling. This color will disappear in a few hours or a few days when the milk has used up the oxygen which entered while the seal was hot and in a liquid state. Litmus reduces over a potential range more negative than that of methylene blue. Milk so treated will also reduce potassium indigo tetrasulphonate and will cause the first irreversible reduction of janus green but will not reduce the pink safranin compound which results, nor will it reduce safranin. The potential ranges over which these dyes reduce are more negative than that of the methylene blue-methylene white reaction.

Other bacteriological media will reduce certain of these indicators if the dissolved oxygen is driven off by such means as heating. Table 3 shows the reducing power of plain and lactose nutrient broth and plain and lactose nutrient agar. Tubes of the different media containing the various dyes were autoclaved at 15 pounds pressure and a reading made immediately. In two

hours another reading disclosed the fact that reoxidation of all the dyes except janus green had taken place during cooling. Doubtless oxygen had entered the media while the vaseline seal was liquid and convection currents possible. This oxygen was consumed by the media after incubation at room temperature for seventy-two hours. The janus green was considered reduced on the appearance of the red safranin compound. This reaction is thought to be irreversible and will be considered in detail in a later paper. The red compound was in no case reduced. Unless protected by a seal, reduced conditions are more easily

TABLE 3

The reduction of various dyes in plain and 1 per cent lactose nutrient broth and plain and 1 per cent lactose nutrient 1.2 per cent agar at pH 6.8 after heating at 15 pounds pressure

	BROTH				AGAR			
	Plain		Lactose		Plain		Lactose	
	Immediately	After 72 hours	Immediately	After 72 hours	Immediately	After 72 hours	Immediately	After 72 hours
2.6 dichloro phenol indo phenol	+	+	+	+	+	+	+	+
Ortho cresol, 2.6 dichloro indo phenol	+	+	+	+	+	+	+	+
Methylene blue	±	±	+	+	+	+	+	+
Janus green	−	±	+	+	+	+	+	+
Litmus	−	−	±	±	±	±	±	+

Note: + means reduced; − means not reduced; ± means partially reduced.

maintained in agar media than in liquid media. Fred reports reduction of methylene blue in all parts of a tube of agar medium upon which a culture was growing, and concludes that the bacteria produced an exoreductase. In the light of present knowledge it seems more probable that the bacteria and the medium consumed the oxygen and that the dye was reduced by constituents of the medium.

The reduction potentials of plain nutrient broth have been studied by Coulter (1928) and Dubos (1929). The latter used the colorimetric method. The former, who measured the po-

tentials electrometrically, found that the initial E_h values of aerated broth lay between +0.15 volt and +0.25 volt, which is remarkably close to the positive E_h limits of fresh milk. The negative potential limits reached were between E_h −0.05 volt and −0.06 volt. The discrepancies between the results of these two investigators appear confirmatory of our observations that the reduction of dyes, when in mixtures with organic complexes such as milk, does not always take place over a potential range identical with the theoretical value for the dye.

Quastel and Wooldridge (1927) advanced the theory that

> the dehydrogenations effected by bacteria are primarily due to polarisations of substrate molecules induced by electric fields which characterise particular centres—the "active centres"—of cellular and intracellular surfaces. . . . . The hypothesis we put forward, that enzymic activity may be regarded as the property of the active centers of cellular and intracellular structures (and this includes the smaller structures capable of extraction from or secretion by the cell) leads to a considerable simplification of the above view. [Note: These authors refer to a popular conception of enzyme behaviour which they had just discussed.] Precisely what enzymic behaviour a particular structure or colloidal aggregate in the cell may possess depends on the nature of the active centres which form a part of the structures or of the colloidal aggregates. . . . . Thus, we may imagine that the protein, nucleotides, etc., are not only so arranged as to form the various substances of the cell but that the arrangement is such that the active centers are formed on these particular substances. Enzymes, therefore, and cellular structures are inseparably connected. . . . . We may regard the entire aggregate as the enzyme, or the particular center as the enzyme.

This conception, though a step in advance of older enzymic concepts, seems inadequate as an explanation of the reduction of methylene blue in solutions containing no cells and no colloidal surfaces, as for instance in sugar solutions, unless we consider the sugar molecules to be in aggregates large enought to provide "active centers" on their surface but small enough to be in true solution. These authors do not report in this paper the potentials of their solutions or the poising effect of methylene blue.

It is impossible, therefore, for a reader to comment on the soundness of some of their conclusions.

Enzymes are believed to be found only where protoplasm exists or has existed. The constituents of the protoplasm of microörganisms are necessarily derived from media. Some of the media have been shown to have reducing properties. The indications are that these reducing properties are imparted to the media by simple and definite chemical constituents. It is reasonable to attribute protoplasmic reductions to chemical compounds as simple and definite as those found in bacteriological substrates. The characteristics usually ascribed to enzymes are met in the common media. If the reduction of dyes in milk is carried on by enzymes, we are at a loss to know their exact function. In the light of present knowledge it is improbable that they are hydrogen donators unless we conceive of them as identical with metabolites. Nor is it any more probable that they act as oxygen acceptors. Barthel's experiments with "synthetic milk" suggest that the milk salts act as catalyzers. Enzymes would, therefore, seem to be superfluous for this purpose. If colloid surfaces are necessary for reduction centers or points, these are already provided by the natural colloids of the milk. Certain it is that, if reduction of dyes in milk is due to enzymes, we must of necessity revise our conception of the heat lability of reductases.

The rôle of bacteria

The relationship of bacteria to methylene blue reduction times in milk has received such an enormous amount of attention that there can be, now, no question of a quantitative relationship. Nevertheless, the function of the bacteria in the reduction test is still a controversial matter. It has been believed by many that reduction of the dye is caused by the bacteria directly. If this is true, then the point of reduction is still undetermined. There seem to be four possibilities in this regard, viz.: (1) Within the bacterial cell, (2) At the surface of the cell, (3) At a distance from the cell due to diffusible substances elaborated by the cell, presumably enzymes, and (4) a combination of any two or all of these.

As we have already pointed out, it is probable that all protoplasm has a reducing power. Needham and Needham (1926), Cohen, Chambers, and Reznikoff (1928), and others have demonstrated such power in the protoplasm of amoeba and other cells. It is reasonable to ascribe a similar property to the protoplasm of the bacterial cell. But it is highly improbable that the major reduction of the dye in the methylene blue reduction test takes place within the bacterial cell or at the surface of the cell. Plate counts of 38 samples of milk taken at the moment of reduction varied from 3.5 million to 45 million with an average count of 21 million bacterial colonies per cubic centimeter. The microscopic picture obtained with a stained smear of such milk shows uneven distribution of the bacteria and is not one which would lead to an expectancy of even disappearance of the dye from the milk, if all reduction were taking place within the cell or at the surface of the cell. In other words, 21 million points of reduction per cubic centimeter seem too few to allow for a conception of homogeneous distribution and disappearance of the dye. We are unable to conceive of a mechanism which would allow such a rapid return of the blue color to the milk on shaking a reduced sample with air if the oxidative process must take place within, or at the surface of, the cell.

Reduction of methylene blue in milk by bacteria has been reported in concentrations as high as 1:3000. In 1 cc. of such milk there would be 0.00033 gram of dye. If we assume that 100 million bacterial cells are present in each cubic centimeter of the milk at the time of reduction and that 100 million bacteria weigh 0.0001 gram, then a much greater weight of dye than of bacteria would be present. It is inconceivable that this amount of dye would concentrate in the living cells. We have already shown that the reducing properties of raw milk are sufficient to account for the reduction of the dye without the aid of bacteria.

All of our work tends to confirm the hypothesis of Barthel (1917) that the disappearance of methylene blue in raw milk takes place in two stages, viz.: (1) the removal of the dissolved oxygen by bacteria; (2) the reduction of the dye by constituents of the milk. It is now amply demonstrated that the milk itself

assists the bacteria in fixing the free oxygen, but this process is so slow as to be of no great importance in the reduction test as ordinarily employed.

TABLE 4

The effect of varying concentrations of dye upon the reduction times of seven samples of milk

CONCENTRATION	TUBE NUMBER	MILK SAMPLES						
		No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7
1:400,000	1			0:45	4:15	6:50		
	2			0:45	4:15	6:50		
1:330,000	1			0:45	4:15	7:15	4:00	
	2			0:45	4:20	7:15	4:00	
1:280,000	1			0:45	4:25	7:25	4:00	
	2			0:45	4:35	7:30	4:00	
1:250,000	1	1:05	2:55	0:45	4:35	7:20	4:15	
	2	1:05	3:05	0:45	4:35	7:50	4:15	
1:220,000	1	1:00	3:00	0:45	4:35	8:00	4:30	
	2	1:00	2:55	0:45	4:35	8:00	4:30	
1:200,000	1	1:05	3:00	0:45	4:35	8:05	Broken	4:30
	2	1:05	3:00	0:45	4:35	8:00	4:40	4:45
1:180,000	1	1:00	3:12	0:45	4:35	8:20	4:50	
	2	1:00	3:00	0:45	4:35	8:05	4:45	
1:160,000	1	1:05	3:00	0:45	4:45	8:35	4:50	4:35
	2	1:07	3:05	0:45	4:45	8:45	4:50	4:45
1:150,000	1			0:45	4:45	8:40	4:50	
	2			0:45	4:50	8:20	4:50	
1:140,000	1			0:45	4:45			4:35
	2			0:45	4:50			4:55
1:120,000	1							4:45
	2							4:55

THE REDUCTION CAPACITY OF MILK

Clark distinguishes between reduction "capacity" and reduction "intensity." The reduction "capacity" of milk is, loosely,

the amount of dye which the milk will reduce when the potential or "intensity" is such that reduction is possible. The standard concentration of methylene blue used in the reduction test is 1 part of dye to 200,000 parts of milk. This concentration has been found to impart a sufficiently deep blue color to the milk to give a comparatively sharp endpoint. The lower the concentration of the dye the less will be the inhibition of bacterial growth. If the reducing capacity of milk is sufficiently large to allow the reduction of dye in a 1:100,000 concentration in the same time as a 1:200,000 concentration, then accurate measurements of the milk samples, within these limits, is not of great importance. Small errors in measurements necessarily occur in

TABLE 5

Reduction times of raw milk with varying dye concentrations, the oxygen being removed by hydrogen or carbon dioxide

DYE CONCENTRATION	REDUCTION TIME
1:200,000	0:30
1:100,000	0:40
1:40,000	2:07
1:20,000	Not reduced in 3 hours
1:10,000	Not reduced in 3 hours

practice. A series of experiments was, therefore, undertaken to determine the effect of such errors. The results of experiments with seven samples of milk to which the dye was added in varying concentrations are given in table 4.

These data show that there may be a slight increase in the reduction time with increase in the concentration of the methylene blue. The concentration must be approximately doubled before any appreciable increase in reduction time is observed. To see if this increase of reduction time is due to the limiting "capacity" of the milk or to an increasing antiseptic effect, a series of tubes containing 10 cc. of fresh morning herd milk of low bacterial content were treated with varying concentrations of methylene blue. They were stoppered but connected by means of glass tubing. After being placed in the water bath washed

molecular hydrogen was bubbled through them in the order in which they are reported in table 5.

Tubes of the same milk were subjected to a similar manipulation after being heated in the autoclave at 15 pounds pressure for one hour. In this case the washed gas was led first through the milk of higher dye concentration, then through the milk of lower dye concentration. Identical results were obtained with the same milks when carbon dioxide was substituted for hydrogen. The results with the heated milk are given in table 6. These data indicate that any considerable increase in the reduction time of a dye concentration of 1:100,000 over that of a concentration of 1:200,000 is due to the antiseptic effect of the dye. This conclusion is in accord with that of Hastings, Davenport, and Wright (1922).

TABLE 6

Reduction times of heated milk with varying dye concentrations, the oxygen being removed by hydrogen or carbon dioxide

DYE CONCENTRATION	REDUCTION TIME
1:100,000	0:20
1:200,000	0:10
1:200,000	0:10

Another sample of milk was autoclaved with varying dye concentrations and 1 part of dye in 1000 parts of milk was reduced, while no reduction was observed when the dye concentration was 1:100. This does not necessarily limit "capacity" to this figure; it is possible that the "poising" action of the methylene blue in these high concentrations is great enough to prevent the falling of the potential through the range of the dye. Whether heating milk increases the reducing "capacity" cannot be said. Dubos (1929) failed to take "poising" action into consideration in attempting to measure the reducing capacity of nutrient broth. His data suggest the influence of this factor. It is evident from these figures, however, that the reducing "capacity" of raw milk is sufficient to take care of double the dye concentration usually employed in the test.

THE RATE OF REDUCTION

Attempts have been made to measure the rate of reduction of methylene blue in milk by titration with titanium trichloride. The investigators using this method have concluded that reduction of the methylene blue begins immediately on addition of the dye to the milk. This is contrary to the common observation that the disappearance of the blue color is usually rapid when once decolorization sets in. In many milks, even in long-time reducing milks decolorizing evenly, the intense blue color remains to within five, ten, or fifteen minutes of complete visual reduction. The reoxidation of the dye on shaking with oxygen is relatively rapid. Our experiments with deaerated milks substantiate the theory that the reduction of the dye in the standard concentration is also rapid.

TABLE 7

Comparison of reduction times when methylene blue was added at the start and near completion of the test

DYE ADDED AT START		DYE ADDED AFTER 12 HOURS	
Number of tubes	Reduction time	Number of tubes	Reduction time
1	12:30	7	12:30
2	12:45	6	12:45
1	13:00	2	13:15
2	13:15	1	13:30
Average.....	12:55		12:46
Variation.....	0:45		1:00

Approaching the problem in a different way, a long-time reducing milk was chosen. To six tubes of this milk the usual dye concentration was added at once and these tubes were then placed in the water bath with sixteen tubes of the same milk to which the dye was added after twelve hours incubation. The results are given in table 7. As will be seen from the table, complete visual reduction was effected in 7 of these tubes within thirty minutes of the time the dye was added. Variation of reduction times in duplicate tubes of milk and uneven disappearance of the dye in the same tube will be given full consideration in a later paper of this series and a theory offered in explanation.

If the reduction of methylene blue in milk is effected by enzymes elaborated by the bacteria, which was the popular theory up to very recent times, it seems necessary to assume that reduction of the dye begins immediately on addition to the milk. The experimental results tabulated above are explainable on the older basis if a concentration of enzymes is built up in the milk. Nevertheless, the question is pertinent, is titanium trichloride specific for methylene blue? The titration curves reported by Fred (1912) are remarkably similar to the potential-time curves obtained by Clark and by ourselves. This suggests that oxygen was being measured. It has been adequately demonstrated that the older hypothesis of dye reduction is untenable. Clark (1925) reports the potential (E_h) ranges within which methylene blue changes from 4 per cent reduced to 96 per cent reduced at different hydrion concentrations. Complete visual reduction is within a narrower range than this. The rate at which methylene blue will reduce in milk depends upon the speed with which the potential ("intensity") passes through this range. Examination of the charts in the cited paper and those reported in this paper will leave no doubt that the time taken for the potential to pass through the range of the methylene blue-methylene white reaction is usually short.

SUMMARY

Potential-time curves of milk and milk methylene blue mixtures are given. The positive E_h limits of all the milks lay between +0.2 and +0.3 volt. The negative potential limits reached by all the milks reported in this paper approximated E_h −0.2 volt, due, it is suggested, to the predominating influence of *Streptococcus lactis*.

The range of reduction of methylene blue in milk has been observed to be more positive than the theoretical value at this pH, and varies considerably in different milks.

An explanation is suggested for the upward trend of the potential curve sometimes noted after the negative limit is reached.

The "poising" effect of methylene blue in the standard concentration in milk is so small as to be almost negligible.

The rôle of hydrogen in reduction is discussed, and the effect of some substances upon reduction times shown, as well as the effect of cysteine upon potentials in milk.

The rôle of oxygen is discussed and evidence presented of its relation to reduction and to reduction potentials.

The necessity for revision of enzyme concepts is made apparent and attention is drawn to the need for consideration of the reducing properties of bacteriological media.

All of our work tends to confirm the theory of Barthel that the disappearance of methylene blue in the reduction test in milk takes place in two stages, viz.: (1) the removal of the dissolved oxygen by bacteria; (2) the reduction of the dye by constituents of the milk.

Evidence is brought forward to show that the reducing "capacity" of raw milk is sufficient to reduce a concentration of methylene blue of 1 part of dye to 100,000 parts of milk in approximately the same time as in the standard concentration, 1:200,000. The "capacity" is probably much greater than this. The "capacity" of heated milk is sufficient to reduce this dye in a concentration as high as 1:1000. We are not in a position to say that heated milk has a greater "capacity" than raw milk.

The rate of reduction of methylene blue in raw milk is rapid and depends upon the speed with which the potential passes through the range of this dye. The time taken for the reduction of the dye in normal raw milk is usually short in comparison with the time necessary for oxygen consumption.

REFERENCES

BARTHEL, CHR. 1917 Weitere Untersuchungen über die Reduktaseprobe, sowei Vergleiche mit einigen anderen neueren Milch hygienischen Untersuchungsmethoden. Zeitschr. Untersuch. Nahr. u. Genussmittel., **34**, 137–164.

BARTHEL, CHR. 1925 Über die Eigenreduktion der Milch. Arkiv. f. Kemi, Mineralogi, O. Geologi., **9**, 19.

BURRI, R., AND KÜRSTEINER, J. 1912 Zur Klärung der Anschauungen über die reduzierenden Eigenschaften der Kuhmilch. Milchwirtsch. Zentralb., **41**, 40–44.

CLARK, WM. M. 1924 Life without oxygen. Jour. Wash. Acad. Sc. **14**, 123–138.

CLARK, WM. M., ET AL. 1925 Studies on oxidation-reduction. VIII. Methylene blue. Pub. Health Rep., **40**, 1131–1201. Reprint 1017.

CLARK, WM. M., ET AL. 1928 Studies on oxidation-reduction. I–X. Hygienic Lab. Bul. 151.

CLARK, WM. M., ET AL. 1926 Studies on oxidation-reduction. X. Reduction potentials in cell suspensions. Supplement No. 55 to Pub. Health Rep.

COHEN, B., CHAMBERS, R., AND REZNIKOFF, P. 1928 Intracellular oxidation. I. Reduction potentials of *Amoeba dubia* by micro injection of indicators. Jour. Gen. Physiol., **11**, 585–612.

COULTER, C. B. 1928 Oxidation-reduction equilibria in biological systems. I. Reduction potentials of sterile culture bouillon. Jour. Gen. Physiol., **12**, 139–146.

DUBOS, RENÉ. 1929 Observations on the oxidation-reduction properties of sterile bacteriological media. Jour. Exper. Med., **49**, 507–523.

FRED, E. B. 1912 A study of the quantitative reduction of methylene blue by bacteria found in milk and the use of this stain in determining the keeping quality of milk. Centralb. f. Bakt., etc., II Abt., **35**, 391–426.

HARDING, T. S., AND CARY, C. A. 1926 Glutathione in blood and its utilization in milk secretion. Proc. Soc. Exper. Biol. and Med., **23**, 319–322.

HARVEY, E. N. 1919 The relation between the oxygen concentration and the rate of reduction of methylene blue by milk. Jour. Gen. Physiol., **1**, 415–419.

HASTINGS, E. G., DAVENPORT, A., AND WRIGHT, WM. H. 1922 The influence of certain factors on the methylene blue reduction test for determining the number of bacteria in milk. Jour. Dairy Sc., **5**, 438–454.

HOPKINS, F. G. 1921 On an autoöxidisable constituent of the cell. Biochem. Jour., **15**, 286–305.

NEEDHAM, J., AND NEEDHAM, D. M. 1926 The oxidation reduction potential of protoplasm. A review. Protoplasma, **1**, 255–294.

QUASTEL, J. H. 1926 Dehydrogenations produced by resting bacteria. IV. A theory of the mechanism of oxidations and reductions *in vivo*. Biochem. Jour., **20**, 166–194.

QUASTEL, J. H., AND WOOLDRIDGE, W. R. 1927 Experiments on bacteria in relation to the mechanism of enzyme action. Biochem. Jour., **21**, 1224–1251.

THORNTON, H. R. 1927 The methylene blue reduction test. Thesis, Library, University of Wisconsin.

THORNTON, H. R. 1929 Reduction of janus green B in milk. Jour. Bacteriol., **17**, 34.

THORNTON, H. R., AND HASTINGS, E. G. 1927 Some aspects of the methylene blue reduction test of milk. Jour. Bacteriol., **13**, 62–63.

THORNTON, H. R., AND HASTINGS, E. G. 1928 Oxidation-reduction potentials in milk. Jour. Bacteriol., **15**, 26.

THUNBERG, T. 1925 Das Reduktions-Oxidations Potential eines Gemisches von Succinat-Fumarat. Skand. Arch. Physiol., **46**, 339–340.

VIALE, G. 1925 Sulle azoni reducenti del latte. Nota III. La presenza de gruppo solfidrilico del latte e la reduzione del bleu di metilene. Studi Sassaresi, Serie II, **3**, 659–662.

WIELAND, H. 1922 Ueber den Mechanismus der Oxydationsvorgänge. Ergebnisse d. Physiol., **20**, 477–518.