SOME FACTORS AFFECTING OXIDATION-REDUCTION POTENTIALS IN DAIRY PRODUCTS

by

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INTRODUCTION

The use of stains as indicators of the reducing power of biological solutions has been employed for many years. These stains, in equilibrium with their reduction products, are used as indicators of oxidation-reduction intensities in a manner comparable to the use of acid-base indicators in hydrogen ion studies. Of the many dyes that may be used, methylene blue has been found to be the most satisfactory for measuring the reduction of biological solutions by microorganisms.

Although the early conceptions of biological reduction processes have been completely reorganized, it is interesting to observe that the selection of the dye and the concentration employed have not been changed by a more fundamental understanding of the factors concerned.

The methylene blue reduction test, as it is used today, is one of the most practical tests for determining the quality of milk. It was early recognized that the reduction of this dye in milk was closely associated with the bacterial population. The mechanism of the reduction of methylene blue in milk has been a much disputed point. The early explanation of the process of dye reduction was based on the assumption that specific (reductase) enzymes were produced. Since most of the bacteria in milk were supposed

to produce an enzyme capable of reducing methylene blue, the time required to change this dye to a colorless compound afforded an index to the bacterial population.

A more recent concept of the mechanism of dye reduction is that advanced by Barthel (1925), that the disappearance of methylene blue in milk takes place in two stages, viz., (1) the removal of dissolved oxygen by bacteria, and (2) the reduction of the dye by constituents of the milk. This concept was given support by Thornton and Hastings, (1929), following a potentiometric study of the reduction of this dye in milk. Much work has been done in the past few years in studying factors influencing the reduction of methylene blue in milk. However, no attempt has been made to correlate these factors with changes in oxidation-reduction intensities as revealed by potentiometric measurements. A study of factors influencing the reduction of methylene blue in milk necessarily involves a study of their effect on the oxidation-reduction intensities of the milk. The potentiometer affords a means of following the potential throughout the entire course of reduction, whereas if the observations are limited to the behavior of a dye, only a small part of the entire course of reduction is revealed.

This paper is a presentation of results obtained in a study of factors influencing changes in oxidation-

reduction potentials in dairy products, and the relationship of these changes to the reduction of methylene blue.

LITERATURE REVIEW

Following a study of the quantitative reduction of methylene blue and the use of this stain for determining the keeping quality of milk, Fred (1912), concludes: "(1) Methylene blue was found to be the most useful stain for measuring reduction by microorganisms; (2) the milk flora shows a strong reducing power; (3) reduction in a newly inoculated culture is directly proportional to the growth of bacteria; (4) the quantitative reduction of methylene blue varies with different types of bacteria, however, each species seems to have a definite reducing coefficient; (5) reductases are formed by the growth of bacteria and do not occur in milk when first drawn. Very probably both intracellular and extracellular products take part in the reduction. (6) The reduction test is of practical importance in determining the keeping quality of milk."

Harvey (1919) states that the rate of reduction of methylene blue by milk and acetaldehyde is influenced by the concentration of oxygen in milk.

Hastings (1919) concludes that some constituent of the milk has a reducing action on methylene blue, and that, "sterile milk exposed to the air will not reduce, since the

oxidizing action of the oxygen is more rapid than the reducing action of the milk." He also concludes that the disappearance of the blue color is dependent on the growth of bacteria.

The first data suggesting that the reducing intensity of bacterial cultures might be measurable in terms of electrode potential were presented by Gillespie (1920). In measuring the reduction potentials of bacterial suspensions and of water-logged soils, he observed a trend toward more negative reducing intensities.

Clark (1920) measured the equilibrium potentials of the systems methylene blue-methylene white and indigo-indigo white. As a result of these studies he established quantitative values for the different reduction intensities indicated by these systems.

Following a study of the significance of anaerobiosis, Hall (1921) showed the decolorization of methylene blue in broth to be dependent upon temperature and the amount of alkali present. Hall also noted that sunlight affected decolorization. The blue color could be restored by bubbling CO_2 or O_2 through the broth. He states that adsorption plays an important role in the decolorization of dyes by porous substances such as animal and plant tissues. Gebhardt (1912) showed that light was capable of bleaching methylene blue, the effect being more intense in the absence

of oxygen. The color returns if placed in the dark in the presence of oxygen. However, if the light used to decolorize the dye consisted of wave lengths longer than 620 millimicrons, the reduction is irreversible.

Hastings, Davenport, and Wright (1922) conclude that the reduction of methylene blue is very intimately connected with the vital processes of the cell, rather than with any extracellular by-products.

Using micro-injection methods Needham and Needham (1925), and also Cohen, Chambers, and Resnikoff (1928) observe that Amoeba proteus and Amoeba dubia, under anaerobic conditions completely reduce all reversible oxidation-reduction indicators, but were unable to reoxidize six of the most easily oxidisable indicators. They conclude that the amoeba maintains a fairly constant reduction potential at a zone lying somewhere between rH 17 and 19.

Barron (1929) states that the oxidative action of methylene blue on living cells belongs to the type of oxidative dehydrations; the dye plays its catalytic role on account of its reversibility and spontaneous oxidability by molecular oxygen without a catalyst. Carter (1928) found methylene blue capable of acting both as a hydrogen acceptor and as a photocatalyst, being decolorized when exposed to visible light in the presence of tyrosine.

Coulter (1928) reports that the removal of oxygen from sterile bouillon by de-aeration or by combination with some constituents of the medium disclosed a reduction intensity corresponding to -0.06 volt. He adds that bacterial respiration is a similar process, but since sterile bouillon may attain the level indicated, the development of this degree of intensity in bacterial cultures cannot be attributed entirely to reductive processes directly dependent upon the action of living cells.

Cohen (1928) states that, "bacterial cultures in broth and synthetic media develop progressively increasing reducing intensities which have been followed electrometrically. Oxidation-reduction indicators, within the limits imposed by chemical reactivity and narrow useful range confirm the time:potential curves. The levels of reduction potentials attained by cultures of different bacteria are more or less different and characteristic."

The reduction potentials of B. typhosus in bouillon, when given access to oxygen show a negative drift, as reported by Coulter and Isaacs (1929). They attribute the effect in the first period to exhaustion of oxygen, and were able to restore the initial positive potential by bubbling oxygen through the medium. The potential of B. typhosus in bouillon does not drift to the negative limits when oxygen is bubbled through continuously.

In 1920 Clark presented a comprehensive basis for interpreting, in terms of electrode potential, the results given by biological reduction of reversible oxidation-reduction systems. Clark, Cohen, and Gibbs (1923) (1924) (1925) (1926) made a quantitative study of the potentials of a large number of the oxidation-reduction indicators including methylene blue, and determined the relative position of these indicators on the potential scale. They presented the time:potential curves of samples of inoculated, bottled, and fresh milk.

Gamman, Cohen, and Clark (1926), by measuring the potentials of cells, extracts, and cultures showed a general correlation between the reduction potential of a cell suspension, the cellular reduction of a dye, and the reduction potential of the same dye as determined in pure solution. They showed also, that different species of bacteria attain different levels of reducing intensity and follow different courses.

Sterile broth, when protected from the atmosphere by a vaseline seal, is capable of reducing a number of dyes including methylene blue, as demonstrated by Dubos (1929).

Thornton and Hastings (1929) observed a very close similarity between the potential:time curves of milk with and without methylene blue. Although the potentials of the zone of visible reduction of methylene blue in milk were

found to be variable, they were always more positive than the theoretical zone in pure solutions of this dye at the same pH. These authors were able to decolorize the dye in milk by de-aeration, and to restore the blue color by aeration. They state that their work tends to confirm Barthel's theory of methylene blue reduction in milk.

It was shown by Fildes (1929a) that the period required for the germination of spores of B. tetani depends mainly on the time required for the medium to reach a suitable reducing intensity. The same writer (1929b) reported that the subcutaneous tissues of a living guinea pig maintain an Eh on the positive side of reduced methylene blue, and that the Eh becomes more negative on death. This Eh is more positive than that required for germination of spores of B. tetani and probably accounts for their failure to germinate when injected into a live guinea pig.

Leeper (1930) reported that cooked meat media when exposed to air was reduced by cultures of two aerobes and five anaerobes. Hewitt (1930) measured the potentials of three cultures in several kinds of medium, and found that, C. diphtheriae and Staph. aureus were usually able to attain more negative reducing intensities than a hemolytic streptococcus.

Whitehead (1930) showed that methylene blue when added to fresh milk of good quality, is reduced in a short

time in the presence of sunlight at 37°C., and that the reaction is not due to an enzyme. He was unable, however, to obtain this reaction with milk from which the fat had been removed. The addition of sodium oleate restored the reducing activities of sunlight.

METHODS

Principles Involved in the Measurement of Oxidation-Reduction Potentials

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.

When one of the regal metals is immersed in a solution containing a reversible oxidation-reduction system or systems, a potential difference is set up at the electrode. This potential can be shown to be dependent upon the proportion of oxidized and reduced forms existing in the solution.

It is not practical to measure the potential difference between the metal electrode and the system under consideration. It is practical, however, to set up two half-cells and measure the difference of the potentials at the two electrodes. One of these half-cells is termed the reference electrode and has a known potential with respect

to the normal hydrogen electrode. The normal hydrogen electrode is defined as a platinized platinum electrode held under one atmosphere of hydrogen and immersed in a solution normal with respect to hydrogen ions. The potential of such an electrode is given the arbitrary value zero. An electrode potential referred to this standard is designated Eh and is measured in volts. The greater the oxidizing intensity of a solution, the more positive is the Eh, whereas a greater reducing intensity is reflected by a more negative Eh. Bacteria in milk develop reducing intensities and these changes in milk have been followed potentiometrically by Clark (1925), and by Thornton and Hastings (1927, 1929).

Experimental Methods

A Leeds and Northrup type K potentiometer was used for the measurement of potential differences. The null point instrument employed was a Leeds and Northrup type R galvanometer. Burnished platinum foil electrodes were used for oxidation-reduction potential measurements. Electrodes one centimeter square were welded to a platinum wire which in turn was fused to copper wire. The platinum wire was drawn through a glass tube and the tip of the tube sealed around the wire at the junction of the platinum wire and foil. The upper edge of the platinum foil was fused into

the tip of the glass tube to make the electrode more rigid. A saturated potassium chloride calomel half-cell was used as the reference electrode. This half-cell has a potential of +0.2452 volt when referred to the hydrogen electrode. Connection was made from the KCl calomel half-cell to the solution under measurement by means of a saturated KCl liquid junction and saturated KCl-agar bridges. By means of suitable switches leads from six electrodes were made to the potentiometer. A knife-switch was placed in the circuit between the electrode switches and the potentiometer. By means of this switch the polarity of the potentiometer could be reversed, thereby making possible, the measurement of solutions whose potentials were positive to the reference electrode.

The dye used was certified methylene blue prepared by the Coleman and Bell Company. The working solutions were prepared by diluting a sterile one per cent solution of this dye.

Unless reported otherwise the water bath was operated at 37°C. $\pm$ 1°. The lower half of the KCl calomel half-cell was immersed in the water bath to stabilize its potential. The volume of the samples employed was either ten or fifty c.c. Each sample container was fitted with a two-hole rubber stopper through which the electrode and the KCl-agar bridge were inserted. In all cases the electrodes and the

agar bridges were immersed to a uniform depth.

The potential readings were taken at sufficiently close intervals to follow the potential drift. The results are presented graphically by plotting the potentials reduced to the hydrogen standard (Eh) as the ordinate against time as abscissa. This method presents the potential changes in the form of a curve, designated as potential:time curve. The Eh in volts is computed in the following manner: In case the observed E. M. F. is negative to that of the reference electrode (+0.2452) it is subtracted from this value. If the observed E. M. F. is positive to the reference electrode it is added to 0.2452 to obtain the Eh. in volts.

EXPERIMENTAL

Preliminary Experiments

Choice of Electrodes. The purpose of the first experiments was to find an electrode that was the most suitable for measuring oxidation-reduction potentials.

Gold foil, gold wire, platinum wire, gold plated platinum, and burnished platinum electrodes were compared. The platinum foil and gold plated platinum electrodes were found to give the most uniform results. Of these two, the platinum foil electrode was selected for these experiments

because of the greater ease of preparing and cleaning. Uniformity in pleting and polishing of the gold plated platinum electrodes is essential to reliable measurements. The difficulties involved could only be justified if more accurate and less variable results were obtained. Parallel trials with the two types of electrodes failed to justify the choice of the gold plated platinum.

It was observed that when several electrodes were placed in a single sample of milk, considerable variation in potentiometric values might be expected during the early part of the reduction process. However, as soon as the potential:time curve begins its characteristic downward trend these electrodes come into close agreement. Any other major change in the oxidation-reduction potentials is likewise uniformly reflected by each electrode. Further irregularities in the readings of the various electrodes may be expected when the negative limits of reduction intensity are reached.

Potential:Time Curves of Market Milk. Among the preliminary experiments, potential:time curves were constructed for a number of samples of market milk held under various conditions. The results of one such experiment are shown graphically in Fig. 1. A sample of market milk was divided into three parts, to two of which was added the standard amount of methylene blue (one part of dye to

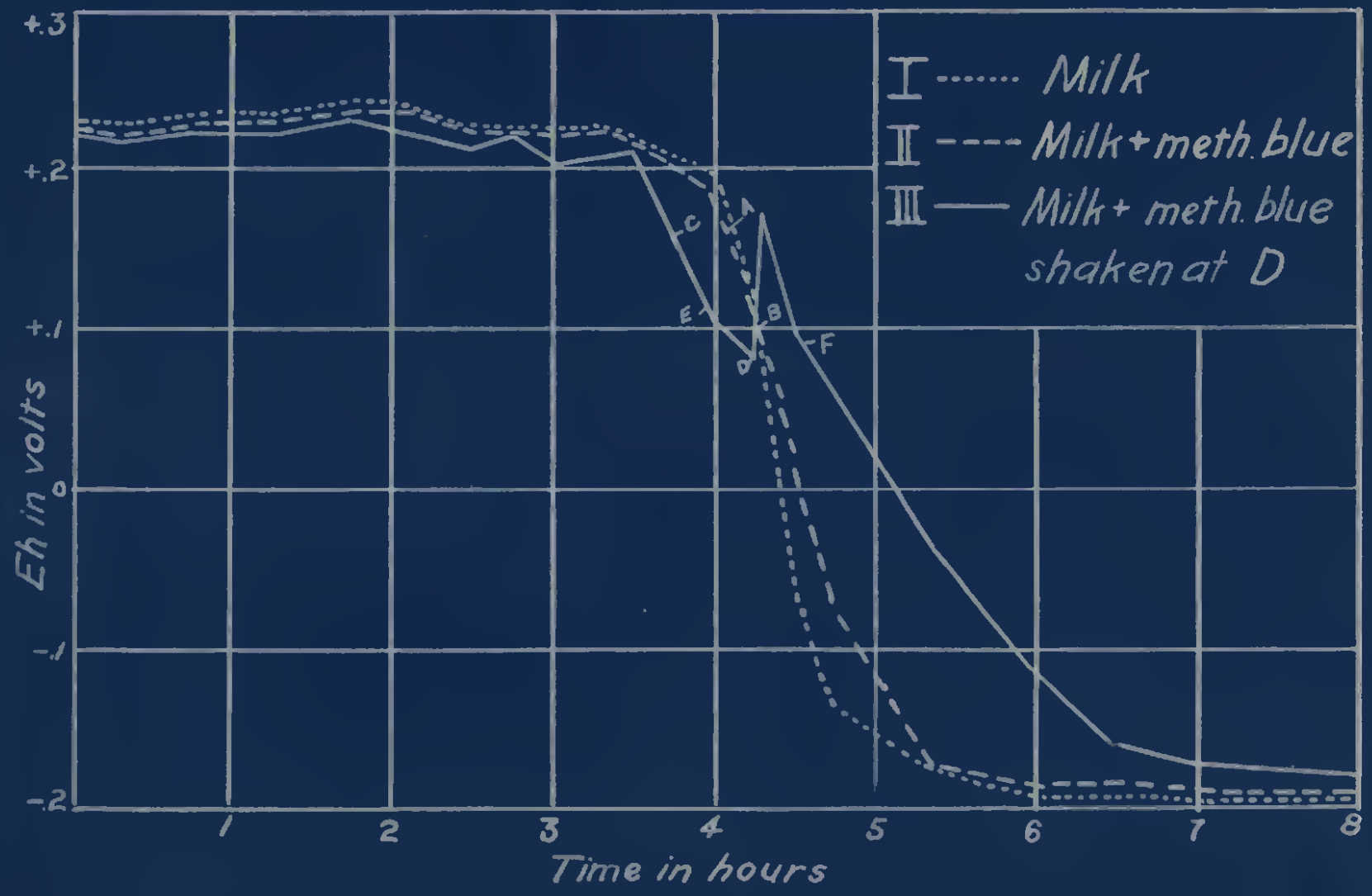

Fig. 1 Potential: time curves of a sample of market milk

200,000 parts of milk). The oxidation-reduction potentials of these three identical samples were followed through the entire course of the reduction process. The curves for the two samples which contained methylene blue (II and III) are represented by the broken line and solid line respectively. The marked similarity in the potential:time curves of samples I and II indicate that the presence or absence of this amount of dye had no material effect upon the trend of the potential. The samples held the initial positive Eh values for approximately four hours and then drifted rapidly toward the negative side. Visible reduction in sample II was first noted at point A and was complete at point B. The zone of reduction was between the Eh limits of +0.15 and +0.1 volt. The negative limit reached was approximately Eh -0.2 volt.

The effect of incorporating oxygen by shaking is illustrated by curve III. The blue color of this sample had completely disappeared at point E, and at point D the sample was shaken vigorously for thirty seconds. This shaking caused the potential to return almost to the original positive values and was accompanied by a return of the blue color. The blue color had again disappeared at point F. The fall of potential from the point F is not so rapid as that occurring in the other two samples. This variation is probably due to the deterring effect of the incorporated oxygen on potential drift.

In further studies, air was bubbled into a sample of milk plus methylene blue after the potential had reached the negative Eh limit of -0.2 volt. The potential returned almost to the positive extreme but the blue color did not return. The potential was observed thirty minutes after the positive extreme had been reached and was found to be falling rapidly to the negative side.

The Effect of the Bacterial Flora on the Form of Potential:Time Curves of Milk

Clark (1935), and Frazier and Whittier (1931a and 1931b) reported that cultures of different species of bacteria run different courses and attain different levels of reducing intensity, thus giving rise to different forms of potential: time curves. Plotting the potential drift of a large number of samples of milk has shown considerable variation in the form of these curves. The curve for a sample of fresh milk is characterized by a rapid fall from the positive to the negative extremes. If a sample of milk giving rise to this form of curve contains the standard amount of methylene blue, the interval between beginning and end of visible reduction will be short, usually less than five minutes. It was commonly observed that the bacterial flora of milk held 48 hours at 3° to 5° C. gave a potential:time curve which fell slowly to the negative extreme. This was accompanied

by a slow decolorization of the methylene blue, frequently observed to extend over a period of thirty minutes. It is evident that a rapidly falling potential will pass through the zone of visible reduction in less time than one which falls slowly, thus explaining the variations in time required for decolorization of the dye in different samples of milk.

The Effect of Fat On the Zone of Reduction of Methylene Blue

In the preliminary experiments, designed to determine the amount of variation that might be expected between different electrodes in the same solution, the oxidation-reduction potentials of cream and skim milk, in addition to those of market milk were measured. These experiments, although designed for another purpose, brought to attention an interesting fact in regard to the potentials of the zone of visible reduction of methylene blue in these three types of solutions.

It was noted that, when the standard amount of methylene blue was added to skim milk, the dye decolorized between the Eh values of zero and +0.05 volt. The potentials of this zone are approximately 0.1 volt more negative than the zone of decolorization of the same amount of methylene blue when added to whole milk. It was also observed that the same amount of methylene blue, when added to cream decolor-

ized between the Eh limits of +0.2 and +0.3 volt. The potential of this zone is approximately 0.1 volt more positive than that observed for whole milk.

To determine more definitely the potential of the zone of reduction of methylene blue in milk of varying percentages of fat, sterile 40 per cent cream, and sterile skim milk were mixed to obtain six solutions containing 40, 30, 20, 10, 5 and 0 per cent of fat. The solutions were inoculated equally with a 24-hour culture of S. lactis and the standard amount of methylene blue added to each. The oxidation-reduction potentials were followed and the potentials of the zone of reduction of the dye observed. The potential :time curves of the six solutions are presented in Figure 2. The potentials of the zone of reduction of the methylene blue are indicated by triangles at the right of the respective graphs. It may be noted that the potentials of the zone of reduction of methylene blue in skim milk are more negative than those observed in the case of cream. Reference to Figure 2 will show that methylene blue was reduced in skim milk between the Eh values of +0.050 and +0.092, and for cream between the Eh +0.245 and +0.275. The zones of reduction in the other samples, without exception, became more positive as the percentage of fat was increased. It is interesting to note that the potentials of the zone of reduction of methylene blue in skim milk approximate more

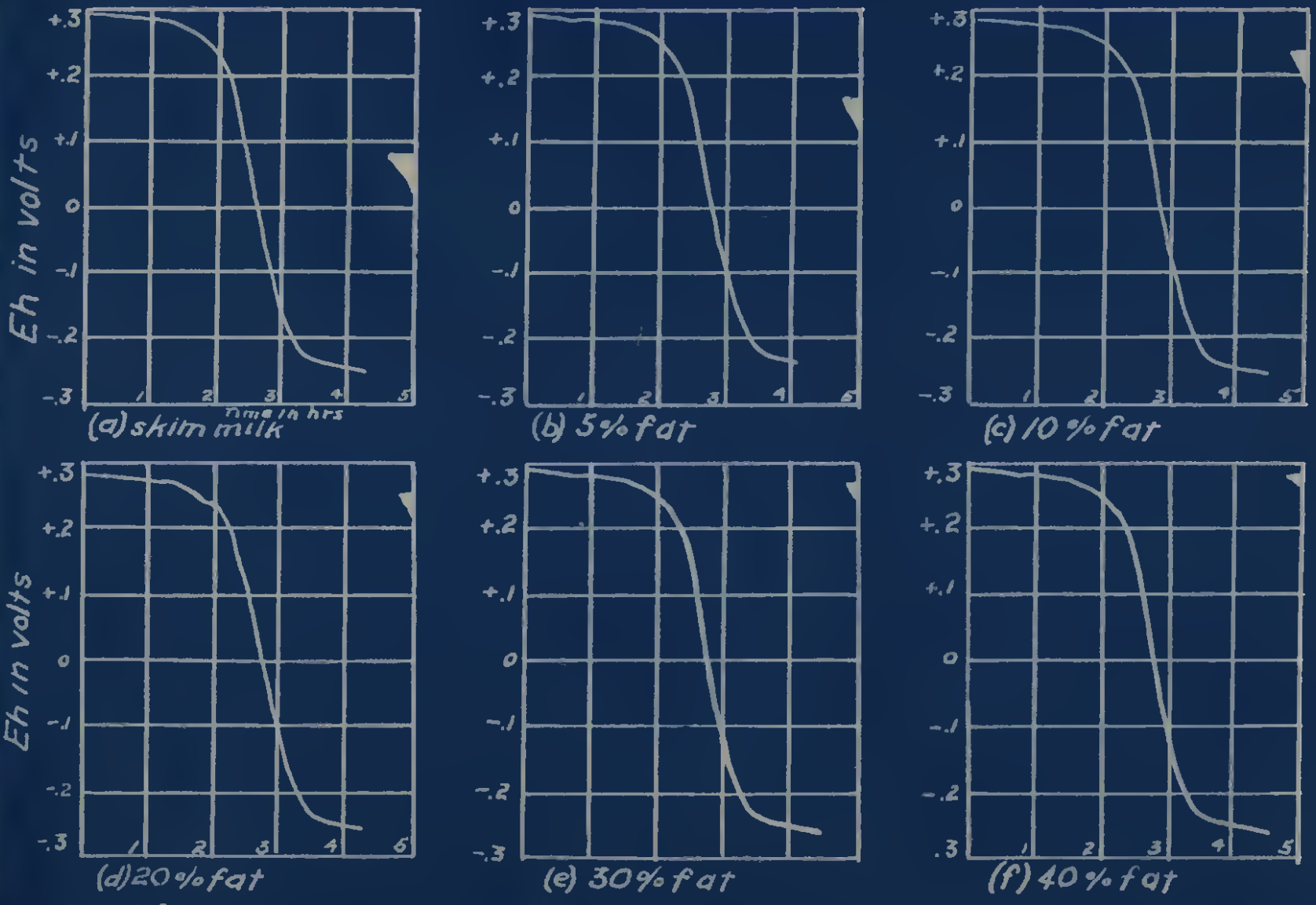

Fig. 2 Effect of varying percentage of fat on zone of reduction of methylene blue

closely the theoretical zone for this dye in aqueous solution as reported by Clark (1925).

The potentials of more than 25 samples of skim milk have been measured, and in no case has the methylene blue reduced at a potential more positive than +0.1 volt. The zone of reduction of methylene blue in 50 samples of 40 per cent cream was never observed to be more negative than +0.225 nor more positive than+0.300 volt.

It may be noted that the form of the potential:time curves is not affected by varying the percentage of fat.

Other factors being equal, it would require a somewhat longer time to reduce methylene blue in skim milk than in 40 per cent cream with the same original bacterial content. In the case of skim milk the oxidation-reduction potential must be carried to the negative limits of approximately +0.05 volt, whereas in the case of 40 per cent cream visible reduction is usually complete at the potential of +0.25 volt.

The exact manner in which fat alters the zone of reduction is not known. Studies of oxidation-reduction phenomena have been limited largely to simple equilibria in aqueous solutions. Many of the fundamental aspects of the simplest systems are yet to be understood. The present status of our knowledge of these simple equilibria certainly does not encourage speculations with respect to complex systems of unknown composition as is the case with biological

fluids. The following explanations of the probable behavior of fat in its effect on methylene blue reduction are presented, therefore, with full cognizence of their purely hypothetical natures.

It is suggested that the addition of an extraneous reversible system such as methylene blue necessitates a readjustment of the equilibrium between this dye and the system already established. On the supposition that there is an equilibrium between the fat and methylene blue, when increasing amounts of fats are added, more and more of the dye would be involved in this equilibrium, and, correspondingly, lesser amounts would be available for colorization.

Another, and perhaps only slightly different conception is based on the assumption that fat adsorbs the dye, thereby removing it from the sphere of activity, in other words the fat may act as a sponge for the dye. Hall, (1921) states that adsorption plays a role as a means of decolorization of dyes by porous substances such as plant and animal tissue.

Experiments were performed in which methylene blue was added to cream at short time intervals, just before and during the period in which the potential was swinging rapidly to the negative side. Although these experiments were not entirely successful, they did show that if adsorption of methylene blue were a factor, the speed of adsorption would necessarily have had to be very rapid. The difficulties en-

countered in determining the end point of reduction during the rapid change of potential, did not warrant definite conclusions from these experiments. Nevertheless, the results presented very little evidence to substantiate the theory based on adsorption of the dye by the fat.

Another, and more tenable explanation of the effect of fat on the zone of reduction is based on the minimum quantity of the oxidized form of the dye requisite to convey a blue color to the eye. When the standard amount of dye is added to skim milk and cream a much lighter color appears in the latter, similarly if dye is added drop by drop until the first tint of blue is evident, considerably more dye is required to bring out the color in cream than in skim milk. Cursory experiments have shown that it requires approximately four times as much dye to give the first perceptible color to 40 per cent cream as for skim milk. This leads to the conclusion that the minimum number of blue molecules necessary to color cream is greater than the minimum for skim milk. When bacterial action in cream reduces a relatively small per cent of the methylene blue molecules to methylene white, this arbitrary minimum for perceptible coloration is soon reached and visible reduction is considered complete. The zone of visible reduction is completed near the top of the curve as indicated in Curve f, Figure 2. In skim milk, however, it is necessary that nearly all of the

dye be converted before visible reduction is considered complete. The zone is correspondingly lower on the curve as indicated in Curve s, Figure 2. If the detection of color is dependent on a requisite minimum number of molecules of the oxidized form of the dye, one would expect the addition of variable amounts of methylene blue to lower the zone for cream to the approximate Eh values observed for skim milk. The results of experiments in which variable amounts of dye were added are presented in Figure 3.

The Effect of Concentration of Dye

Zone of Visible Reduction. The zone of reduction of cream and skim milk may be moved up and down the potential: time curve at will by the addition of variable quantities of dye. In Figure 3 curves A, B, C, and D are representatives of many experiments to determine this point.

Curves A and B show the zone of reduction of methylene blue in skim milk and cream respectively when the standard amount of dye (1:200,000) is added.

By adding only 1 part of dye to 16,000,000 parts of skim milk the zone was changed to approximate that of cream (curve B). Similarly, curve D shows that the addition of 1 part of dye to 10,000 parts of cream caused the zone of reduction to approximate the Eh limits which apply to skim milk when the standard amount of dye is added.

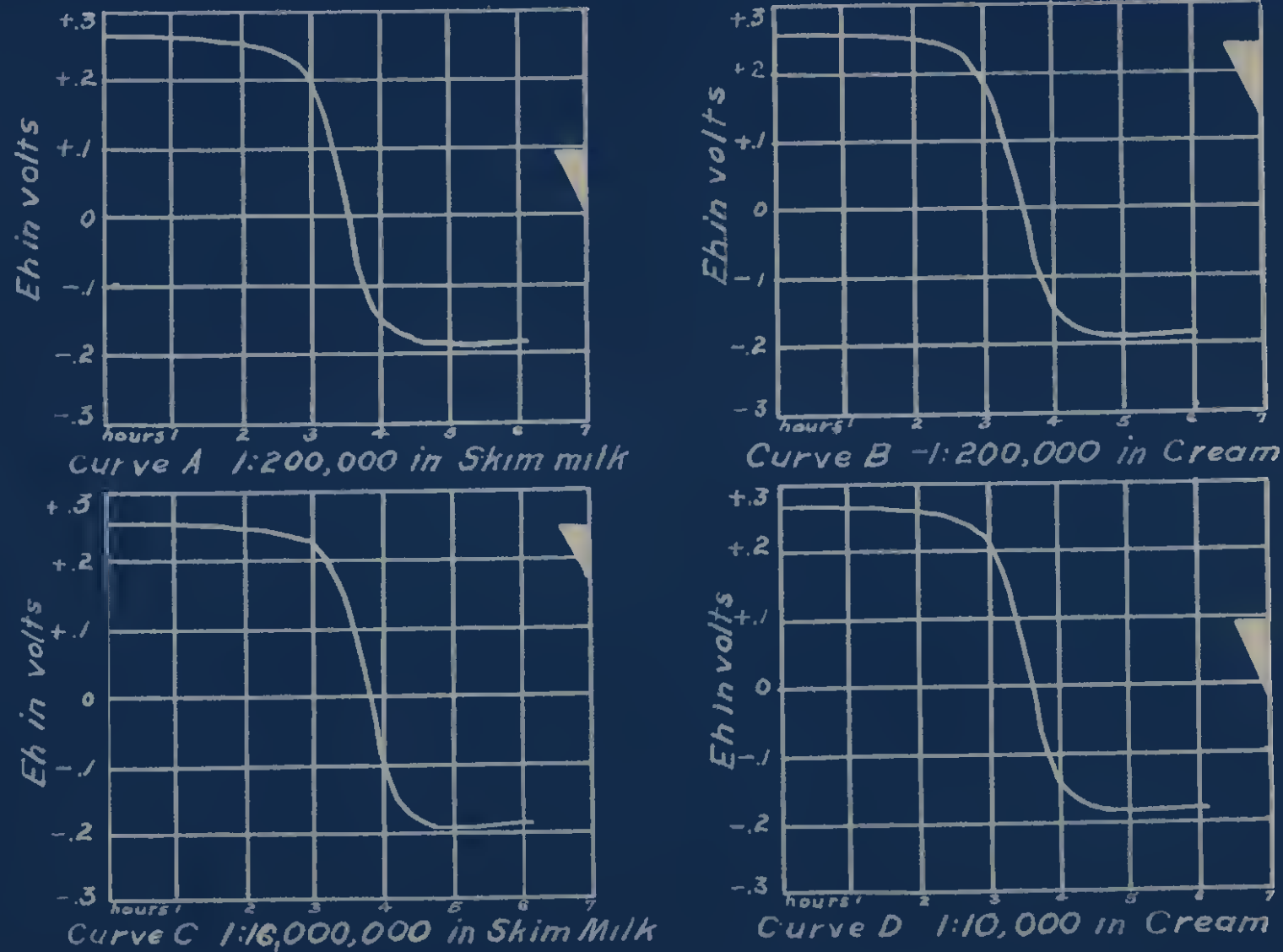

Fig.3 Relation of Concentration to Zone of Reduction of Meth. Blue

Reduction Time. In recent years there has been some controversy in regard to the effect of varying concentrations of dye on the reduction time of milk. Three portions of a sample of milk containing the following concentrations of methylene blue were studied potentiometrically, (a) 1:400,000, (b) 1:200,000, and (c) 1:100,000.

The potential:time curves of these three samples and the zones of reduction are shown in Figure 4. The form of the three curves is alike and would superimpose if plotted upon the same ordinates. The zones of potential within which the methylene blue is reduced are shown by means of triangles. It may be noted that the position of these zones varies with the concentration of dye. In curve b representing the sample containing the normal concentration of dye, it will be noted that decolorisation took place in the zone between +0.165 and +0.235 volt, and was complete after 75 minutes incubation. Curve a represents the sample containing one-half the normal amount of dye (1:400,000). The zone of decolorisation was 0.075 volt more positive than when the normal concentration of dye was used (curve b). Coincident with the more positive zone, the reduction time was shortened from 75 to 55 minutes. Curve c shows the effect of adding twice the normal concentration of dye (1:100,000). The zone of decolorisation of methylene blue in this sample was 0.9 volt more negative than for the sample containing the

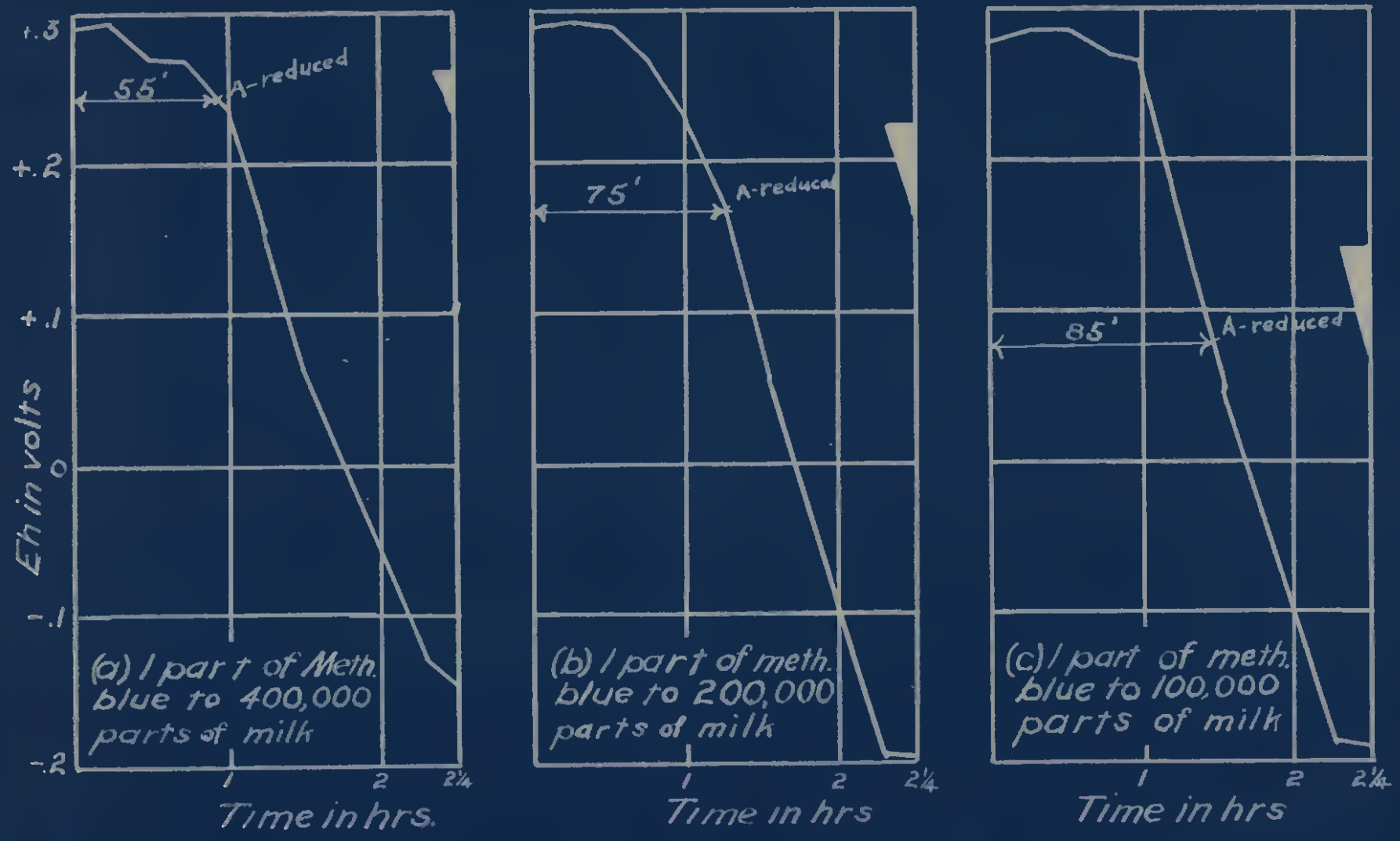

Fig 4 The Effect of Varying Concentration of Meth. Blue on Reduction Time

normal concentration of dye. The time required for the reduction of the dye was increased to 85 minutes as compared with 75 minutes for sample b.

The significant aspect of these three potential:time curves is the potential of the zone of decolorization of the varying concentrations of methylene blue. If it be assumed that the color disappears when less than an arbitrary minimum number of molecules of the blue dye are present, the explanation of the effect of the varying amounts of dye on reduction time becomes simple. If larger than the normal amounts of dye are present, more negative potentials must be reached before decolorization is effected, and hence longer time is required. Similarly, less time would be required to attain the slightly negative potential necessary to effect decolorization of sample a illustrated in Figure 4. In other words the more dye there is present, the longer time required to reach a potential sufficiently negative to diminish the quantity of the dye in the oxidized form below the amount requisite for coloring.

In the concentrations employed (Figure 4) the dye does not affect the course of potential change as is evidenced by the similarity of the three curves.

Form of Potential:Time Curve. The studies in the preceding experiments on the relationship of the concentration

of dye to other factors were confined for the most part to higher dilutions of methylene blue. In the following experiment the effect of more concentrated solutions of dye has been studied. The curves in Figure 5 show the potential drift of four portions of a sample of 20 per cent cream conteining the following concentrations of methylene blue: (1) no methylene blue; (2) 1:200,000; (3) 1:10,000; and (4) 1:5,000. The zones of potential within which the methylene blue reduced are indicated by the letters B (began) and C (completed). The curves of samples 1 and 2 are similar to those in Figure 4, and show that the addition of the normal amount of methylene blue does not alter the form of the potential curve. The zone of reduction of the dye in sample 2 was between the Eh values of +0.2 and +0.24 volt. The potentials of this zone are similar to those previously observed (Figure 4) for 20 per cent cream conteining the normal amount of dye.

The potential curves of samples 3 and 4 illustrate clearly the effect of adding excessive amounts of methylene blue. There are several significant aspects of these four curves which not only show the effect of the addition of excessive amounts of dye, but possibly throw some light on the mechanism of dye reduction in milk.

In the first place it may be noted that all four samples began their swing toward the negative potentials simul-

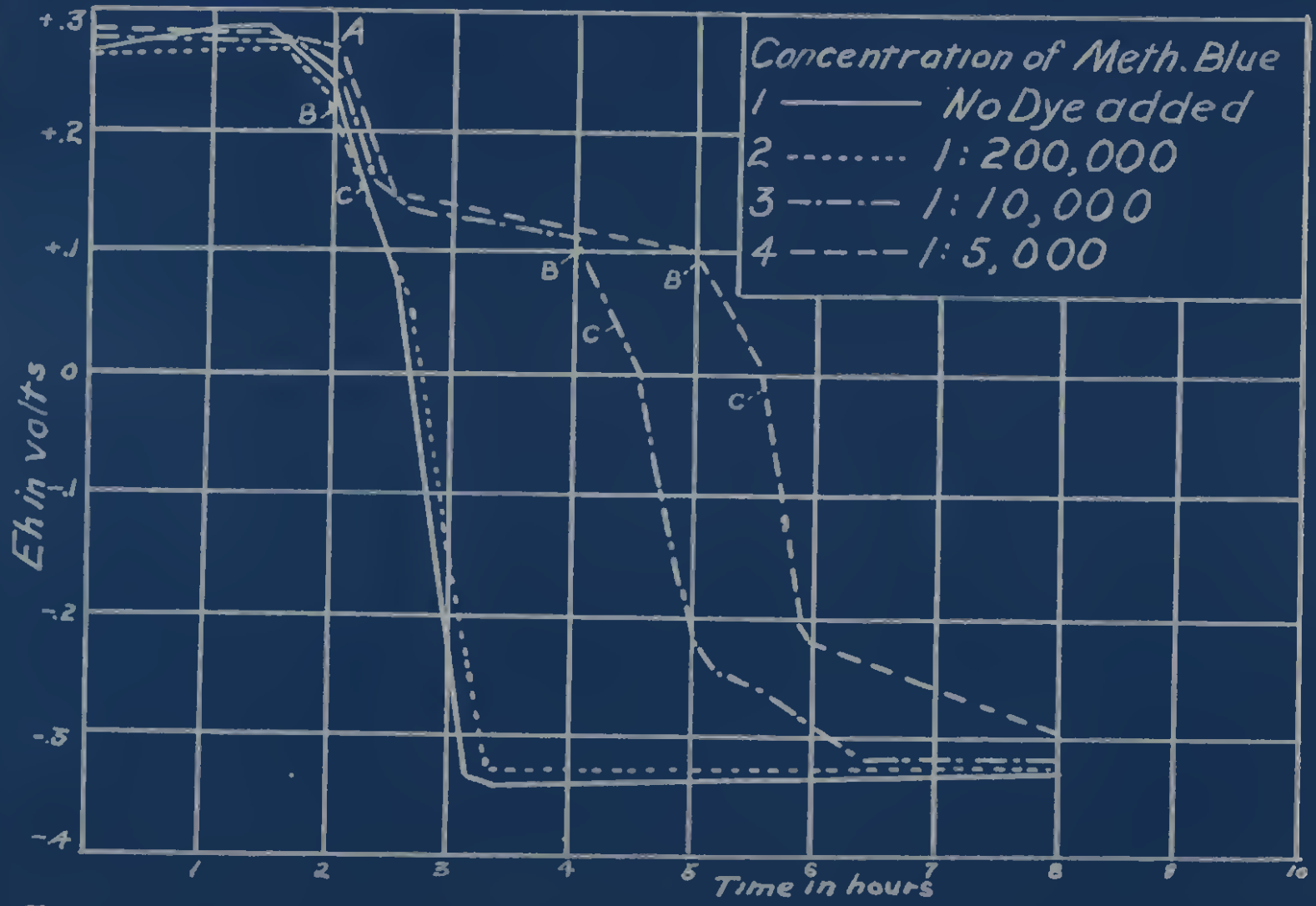

Fig. 5 Potential:time Curves of a Sample of Cream Containing Varying Concentrations of Methylene Blue.

taneously. Since the initial fall in potential is the direct result of bacterial activity, this indicates quite clearly, that at least the highest concentration of dye employed did not exert any antiseptic action.

The plateaus observed in curves 3 and 4, especially when contrasted with the total absence of a plateau in curve 2, emphasize the fact that the poising effect of the dye is directly dependent upon the amount of dye added. Clark defines poising as follows, "A solution may be said to be poised when it tends to resist a change in Eh on addition of an oxidizing or reducing agent."

As the four samples of milk began their initial swing (point A) toward negative potentials, they followed the same general course until they came well within the zone of reduction of methylene blue. The potential drift was not impaired in samples 1 without dye, or in sample 2 in which the normal concentration of 1:200,000 was employed. In samples 3 and 4, however, the large amounts of methylene blue added exerted poising effects which were directly related to the quantities of dye added.

Since the normal concentration of dye employed in the reduction test does not materially affect the oxidation-reduction system, the methylene blue simply serves as a visible indicator that this swing toward more negative potentials has taken place. As the visible reduction occurs

shortly after the swing toward more negative potentials begins, the loss of color of the dye indicates that the bacterial activity has overcome the poising effect of the oxidation-reduction systems of the milk. (Point A has been reached).

The time required for visible reduction became progressively greater as the concentration of dye was increased. For the samples reported in Figure 5 the reduction times and dye concentrations were as follows:

(2) 1:200,000--128 minutes

(3) 1: 10,000--250 minutes

(4) 1: 5,000--335 minutes

It is interesting to note that the reduction of dye in samples 3 and 4 was not completed until after the second drop in the potential had started. The data in Figure 5 further substantiate the observations made in connection with Figure 3, viz., that the amount of dye employed affects the zone of reduction.

The Effect of Varying Concentrations of Sugar on Oxidation-Reduction Potentials in Cream

There is a demand at the present time for a practical test for determining the quality of dairy products such as ice cream and ice cream mix. Early in the course of these experiments, attempts to follow the course of the potential

drift of ice cream showed that the curve tended to pass slowly toward negative values. The visible reduction of the dye was correspondingly delayed over an extended period. Attempts to determine the cause for the peculiar nature of the curve lead to a series of experiments to demonstrate the effect of sugar on the potential drift. Figure 6 shows the results of a typical experiment.

Sterile cream, skim milk, and a cane sugar solution were combined in suitable proportions to give variable concentrations of sugar (0, 10, 20, and 25 per cents) and a constant fat content of 20 per cent. Each sample was inoculated with a 24 hour culture of S. lactis and the standard amount of dye added. The form of the potential:time curves was markedly altered by increasing the percentage of sugar. Increasing the amount of sugar delayed the potential trend to more negative values, which in turn lengthened the reduction time. Although equal amounts of inoculum were added to each sample obviously, the number of bacteria added could not be accurately controlled. Nevertheless, the time required for reduction was directly increased with larger amounts of sugar. The reduction times for the samples in order of increasing amounts of sugar were 155, 165, 215 and 248 minutes respectively. The differences in the form of the potential curves were due, perhaps, to a change in the metabolic activities of the cells, although evidence to support this explanation

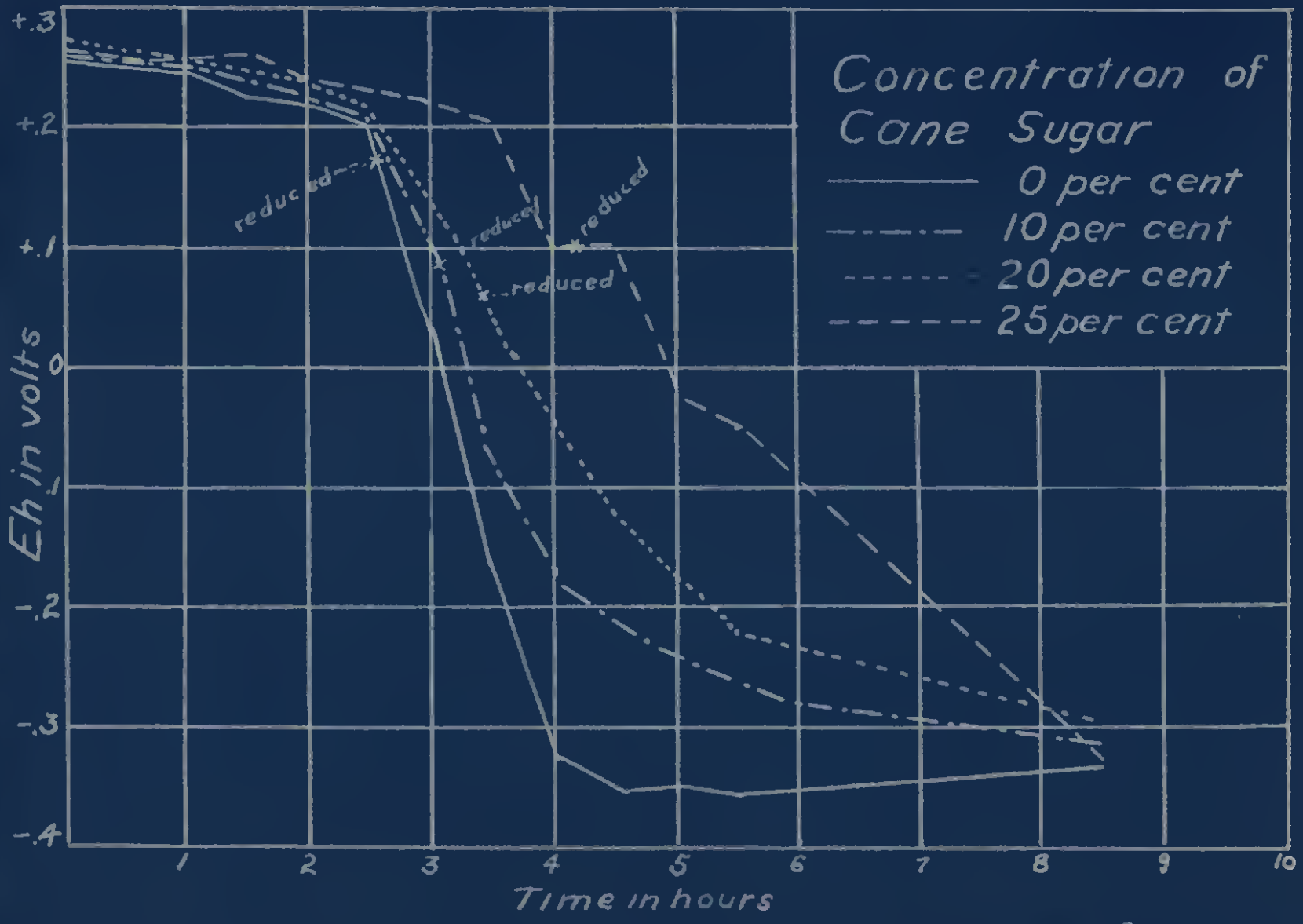

Fig.6 Potential: Time Curves of a Sample of Cream Containing Varying Concentrations of Sugar

is not available. It has been shown by Hewitt (1930) that changes in the medium effect the reduction intensities attained by bacterial cultures. It is of interest to note the extreme negative levels (-0.3 and -0.35 volt) attained by these cultures. Clark (1925) has shown that cultures of S. lactis in milk usually reach a negative limit approximating -0.2 volt.

Effect of Sunlight

It has been known for a number of years that the reduction of methylene blue in milk may be brought about in three ways: (1) living bacteria, (2) an enzyme, catalase, that will reduce methylene blue in the presence of an aldehyde, and (3), according to Whitehead (1930), milk that has been heated to more than 100°C. will reduce methylene blue.

Whitehead also reported the reduction of methylene blue in milk exposed to sunlight, but found that this reaction did not take place in the absence of fat. However, he observed that the addition of sodium oleate restored the ability of fat-free milk to bring about this change, and concluded that fat was essential for the reduction process by sunlight. Preliminary experiments have confirmed most of Whitehead's observations with one exception which will be discussed later.

Procedure. In order to determine the influence of fat on reduction of methylene blue by light, several experiments were performed with milk and cream containing varying amounts of fat. In each case the samples were divided into two parts, one of which was exposed to strong sunlight and the other was protected by wrapping the container in several thicknesses of heavy black paper. In order to maintain a temperature of 37°C., the tubes were submerged about one inch in a water bath held at 41°C. Due to the prevailing low temperature it was not feasible to place the water bath in the open window, therefore the sunlight was filtered through the window pane in addition to the walls of the test tubes.

It was consistently observed that the tubes exposed to sunlight reduced within 15 to 90 minutes, the time being dependent upon the intensity of the sunlight. It was also generally observed that increasing per cents of fat shortened the time required for reduction of the dye by light. Similar results were obtained by adding increasing amounts of sodium oleate to skim milk.

In order to obtain a more complete history of the changes occurring in milk, cream, and skim milk exposed to sunlight, the oxidation-reduction potentials of a number of samples were followed.

Market Milk. Ten cc. samples of market milk were placed in each of four sterile test tubes and the standard amount of methylene blue was added to two of them. One tube of milk containing methylene blue and one without dye were placed in the sunlight; the two remaining tubes were covered with a sleeve of heavy black paper. The oxidation-reduction potentials were measured at suitable intervals. In Figure 7 it may be noted that the potentials of the samples exposed to sunlight became more negative immediately after exposure. This negative drift continued until an Eh value of approximately zero was reached. The milk containing the methylene blue was completely reduced at an Eh value of +.065 volt (point C_1). After the initial rapid fall the potentials remained at an Eh value of approximately zero for three hours or until four hours after the start of incubation. At this time the potentials of the four tubes came into close agreement. The potentials of the two tubes not exposed to sunlight had retained their initial Eh values for three hours at which time they began to fall rapidly to the negative side. The potential drift of these two tubes of milk in the dark may well be attributed to bacterial activity.

Attention is called to the fact that the four curves (Figure 7) tend to converge at an Eh value of approximately zero. It will be noted that this is considerably more posi-

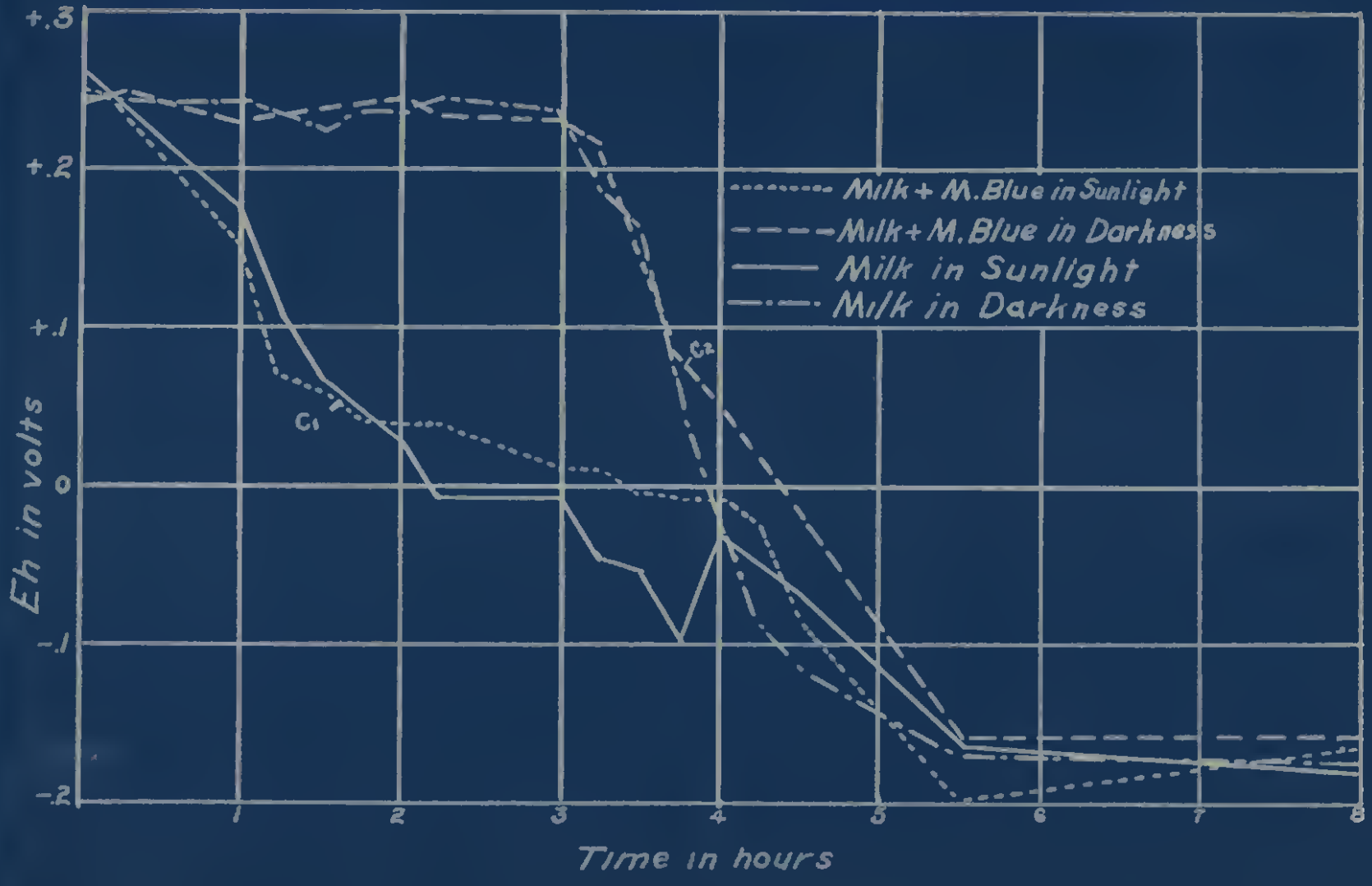

Fig. 7 Potential: Time Curves of Market Milk in Sunlight and Darkness

tive than the ultimate negative limit of the potential drift (-0.2 volt). A comparison of these curves shows quite clearly that the light was unable to lower the potential below the Eh value of approximately zero. After these curves converged with those of the two tubes kept in the dark, they remained in close agreement throughout the remainder of the reduction process. The reduction of the sample in the light preceded that of the sample in the dark by two and one-half hours.

It will be observed that the samples in the dark and in the light showed complete visible reduction (points, C_1 and C_2) at approximately the same Eh values. This suggests that the reduction of the standard quantity of methylene blue (1:200,000) in milk takes place within a definite potential zone, and that the change of color occurs whenever this potential is reached, whether the potential drift be induced by physical or biochemical processes.

Cream. The effect of sunlight on the oxidation-reduction potentials of four portions of a sample of 40 per cent cream with and without dye were studied in the same manner as in the preceding experiment. The potential:time curves are shown in Figure 8. As was observed with milk, sunlight induced a negative potential drift immediately after exposure, whereas the Eh value of the samples in the dark remained constant for several hours. In this experiment, how-

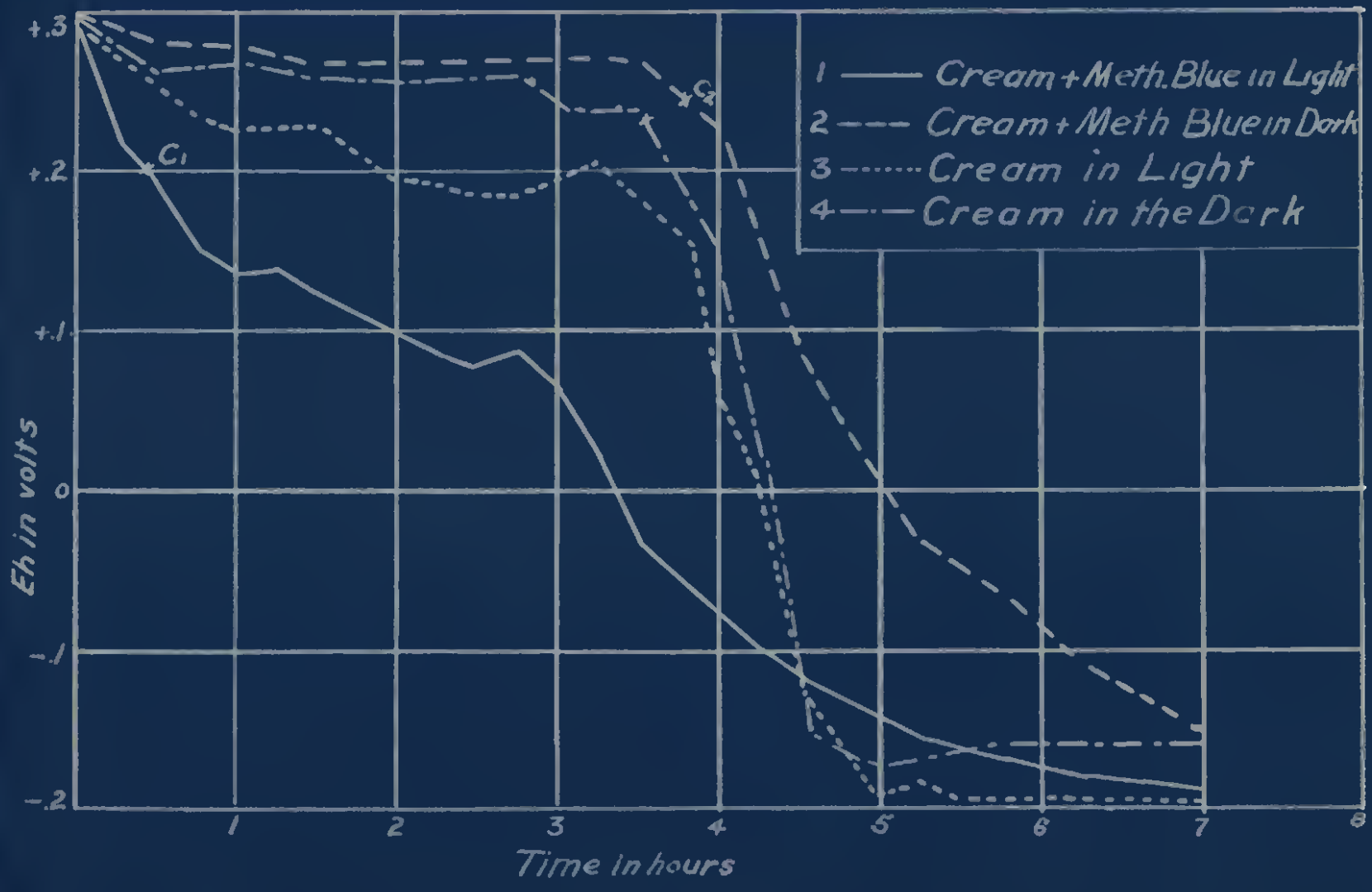

Fig. 8 Potential: Time Curves of Cream in Sunlight and in the Dark

ever, the presence of higher concentrations of fat apparently affected the ability of the light to induce more negative Eh values. It will be noted that the initial potential drift in the two samples (with and without dye) exposed to sunlight is extended over a considerable period, in contrast to the rather sudden fall observed for whole milk (Figure 7). Also, a comparison of curves 1 and 3 shows that when methylene blue is present, sunlight is able to induce more negative potential values than when no dye is added to the cream.

As in the preceding experiment, the potentials of the zones of visible reduction (points C_1 and C_2) are essentially the same for samples in the light and in the dark.

Skim Milk. The oxidation-reduction potentials of skim milk with and without dye exposed to the light and in the dark were followed in exactly the same manner as for cream and whole milk. The potential:time curves of the samples of skim milk are shown in Figure 9. The curves are similar in a general way to those presented in Figures 7 and 8 for cream and whole milk. However, sunlight causes a greater and more rapid fall in potential in the skim milk than in either cream or whole milk. As in the case of cream, the addition of dye to the skim milk enabled the sunlight to induce a more negative potential drift than in the same skim milk without dye. In harmony with previous observations in

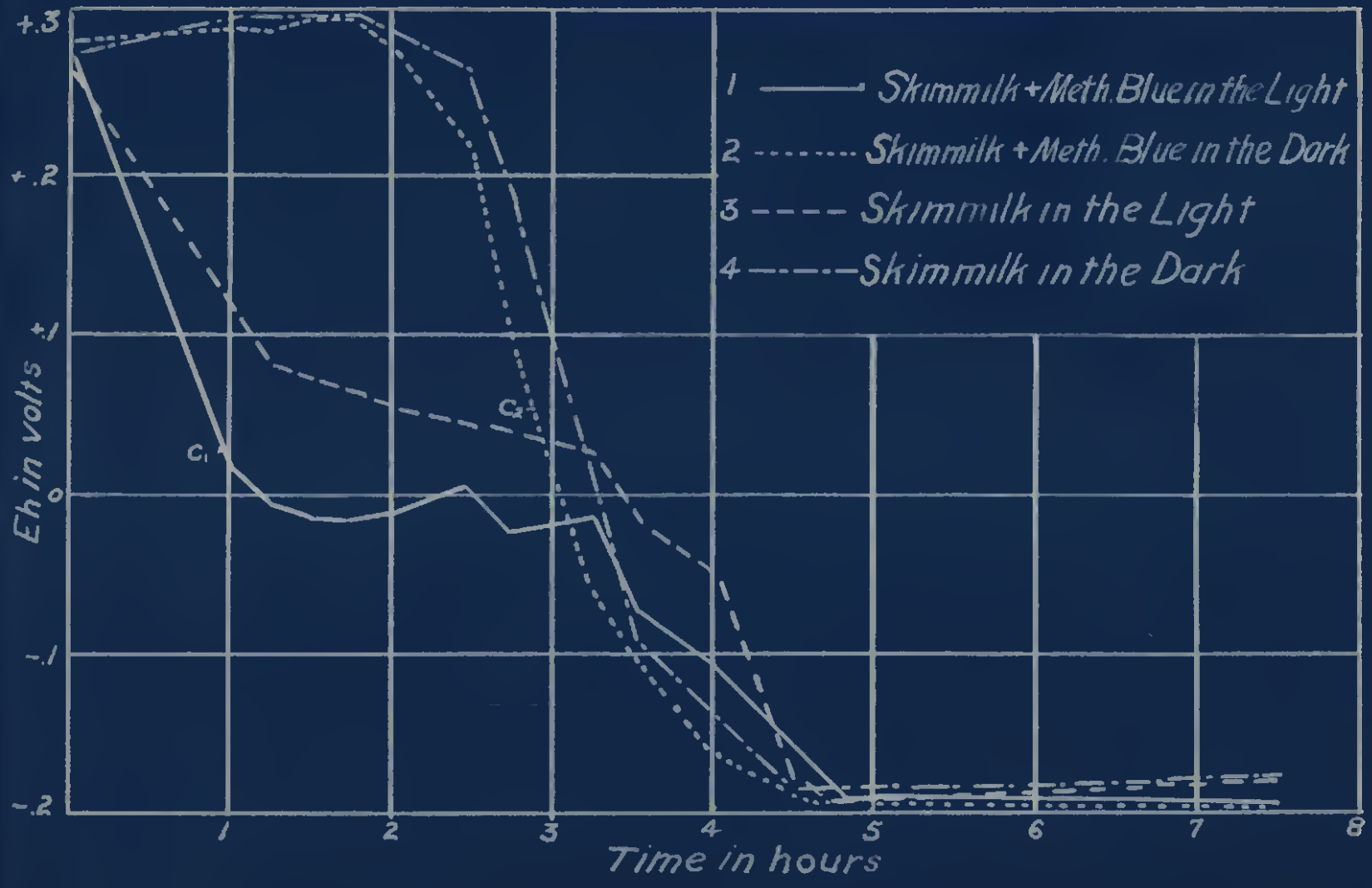

Fig. 9 Potential: Time Curves of a Sample of Skimmilk With and Without Methylene Blue in the Light and in the Dark

Figures 7 and 8, the Eh values for complete visible reduction (C_1 and C_2) were essentially the same, whether induced by bacteria or sunlight. The potentials of the four samples came into close agreement after three and one-half hours and remained together during the remainder of the reduction course.

Skim Milk Plus Sodium Oleate and Sodium Stearate. In the preliminary studies on the reduction of methylene blue by sunlight, it was observed that skim milk containing sodium oleate and methylene blue was readily reduced. In order to determine the effect of such substances on the potential of the zone of reduction of methylene blue, a sample of skim milk was divided into three parts and treated as follows: (1) one per cent sodium oleate, (2) one per cent sodium stearate, and (3) not treated. The standard amount of methylene blue was added to each of the three samples. The potential:time curves and points of complete visible reduction of the three samples are shown in Figure 10. The potentials of the samples containing the fatty acid salts drifted toward the negative side more rapidly than that of skim milk. The potentials of these two samples (1 and 2) dropped rapidly to Eh -0.025 volt, after which it remained fairly constant. After four hours of incubation no more sunlight was available and the potentials drifted to more positive Eh values. The potentials remained in close agreement throughout the

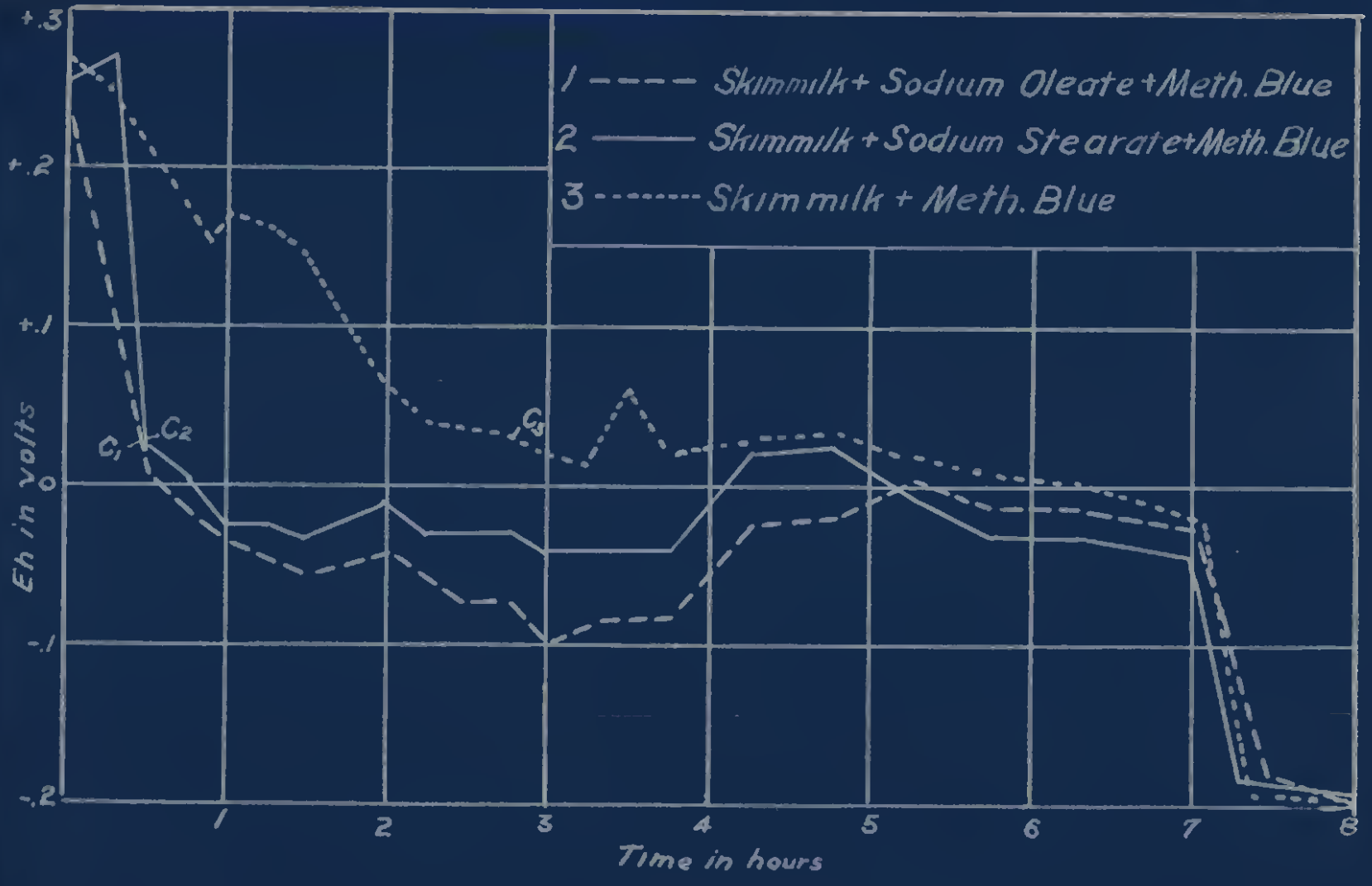

Fig. 10 The Effect of Sunlight on the Potential Time Curves of Skimmilk containing Sodium Oleate and Sodium Stearate

remainder of the reduction process. After seven hours, bacteriel reduction took place as the potentiel of each sample fell to the negative limit of approximately -0.2 volt.

C_1, C_2, and C_3, represent the points at which the methylene blue was completely decolorized. The zone of reduction of methylene blue is evidently not affected by the presence of either of the fatty acid salts employed in this experiment. The uniformity of the Eh values at the time of reduction of the dye in the three solutions (C_1, C_2 and C_3) not only emphasizes this fact, but conforms to the values previously observed for the zone of reduction of this dye in skim milk (+0.025 volt). Any deterring influence which butter fat may have exerted on changes in potential in the preceding experiments, apparently is not induced by one per cent of sodium oleate or sodium stearate.

It was observed in a preceding experiment (Figure 8) that the presence of butter fat accentuated the effectiveness of light as a reducing agent. It is interesting to note that sodium oleate and sodium stearate exert a similar effect.

Relation of Dye to Potential Drift. The results of previous experiments indicate that the presence of methylene blue accelerates the potential change in cream and skim milk when these solutions are exposed to sunlight. In order to study more fully the role played by methylene blue in this

reaction, skim milk containing one per cent sodium oleate was divided into six parts, and methylene blue was added as follows: (1) no dye added, (2) 1:400,000, (3) 1:200,000, (4) 1:100,000, (5) 1:50,000, (6) 1:25,000. The tubes were placed in the water bath and exposed to sunlight. The potential curves and points at which visible reduction was completed are presented in Figure 11.

It may be noted that the potentials of all the samples not only drift to more negative values upon exposure to sunlight, but that with the exception of sample 6 (1:25,000), the fall of potential is directly related to the concentration of dye. The potential of sample 6 does not reach the negative limits attained by samples 4 (1:100,000) and 5 (1:50,000). The most marked difference observed was between samples 1 (no dye) and 2 (1:400,000). It is quite evident that the presence of only a small amount of dye greatly accentuates the potential change induced by sunlight. The accelerating action of the dye is not proportional to the amount of dye added. The addition of methylene blue in higher concentrations than 1:200,000 did not materially increase the reducing intensities induced by sunlight. The potentials of all the samples remained fairly constant after the initial drift toward the negative side. After five hours incubation the potentials dropped rapidly to more negative limits. These latter changes in potential are due,

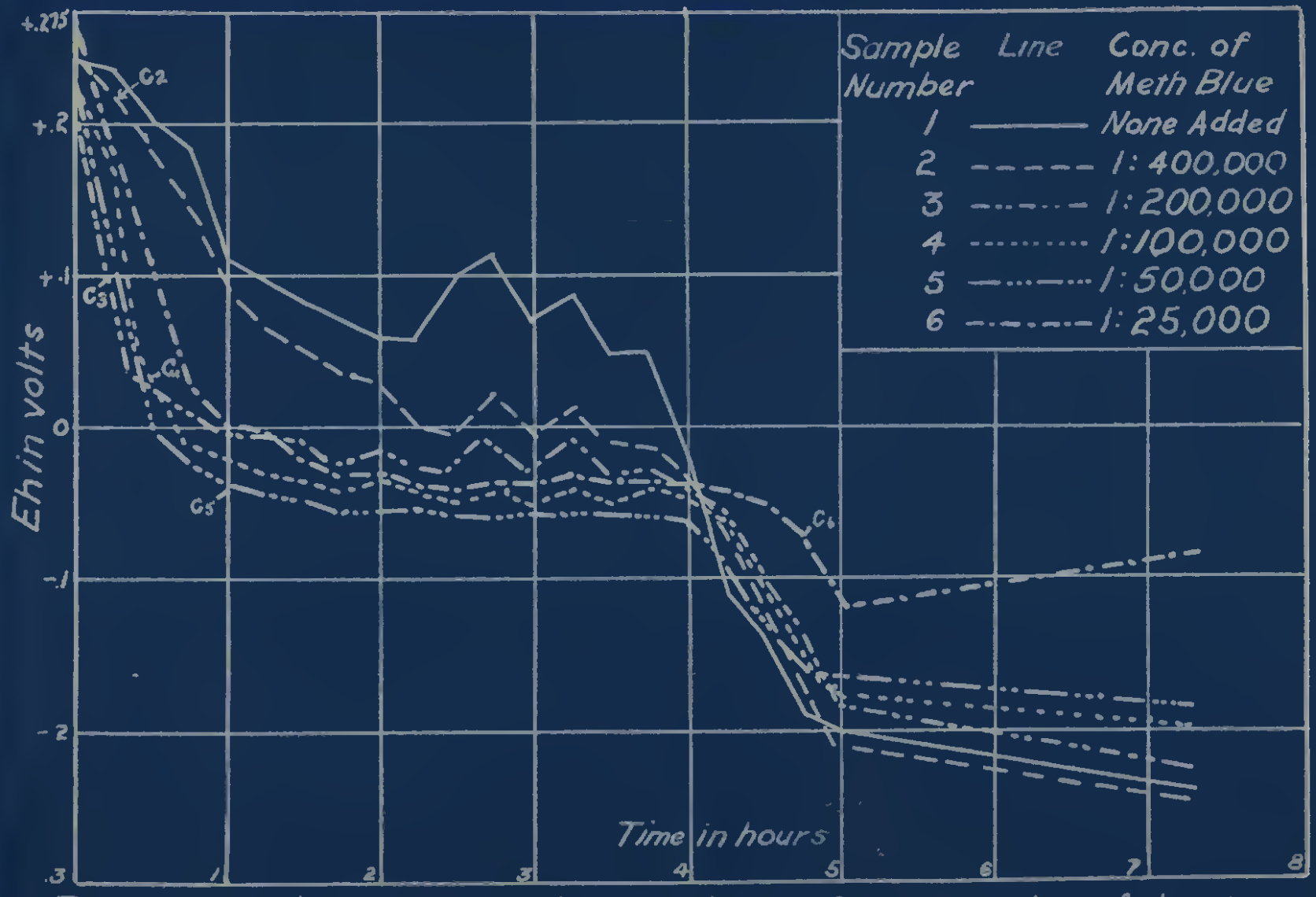

Fig. 11 Potential: time curves showing relation of concentration of dye to potential changes induced by sunlight

no doubt, to bacterial activity.

In Figure 11 it may be observed that the final negative limits attained by these samples, with one exception, are inversely related to the concentration of dye added. The exception noted is sample 1, the negative limit of which is slightly more positive than that of sample 2. The time required for visible reduction for the six samples, increased directly with the amount of dye added. The time varied from 15 minutes for sample 2 (1:400,000) to 285 minutes for sample 6 (1:25,000). The Eh values at which visible reduction of the dye was complete, (C_2, C_3,..C_6) became more negative with each increase in concentration. Sample 6 was not completely reduced by the sunlight. Though lighter in shade, some color was still discernible at the time sunlight was no longer available. Decolorization of this sample was effected, only after bacterial action had induced more negative limits of reducing intensities.

Effect of Alternate Light and Darkness. Figure 12 illustrates the changes in oxidation-reduction potentials induced by alternately placing a solution in the light and in the dark.

Samples of skim milk and market milk were each divided into two portions and methylene blue (1:200,000) was added to one of each. These four samples were exposed to sunlight adjacent to a closed window. The temperature was maintained

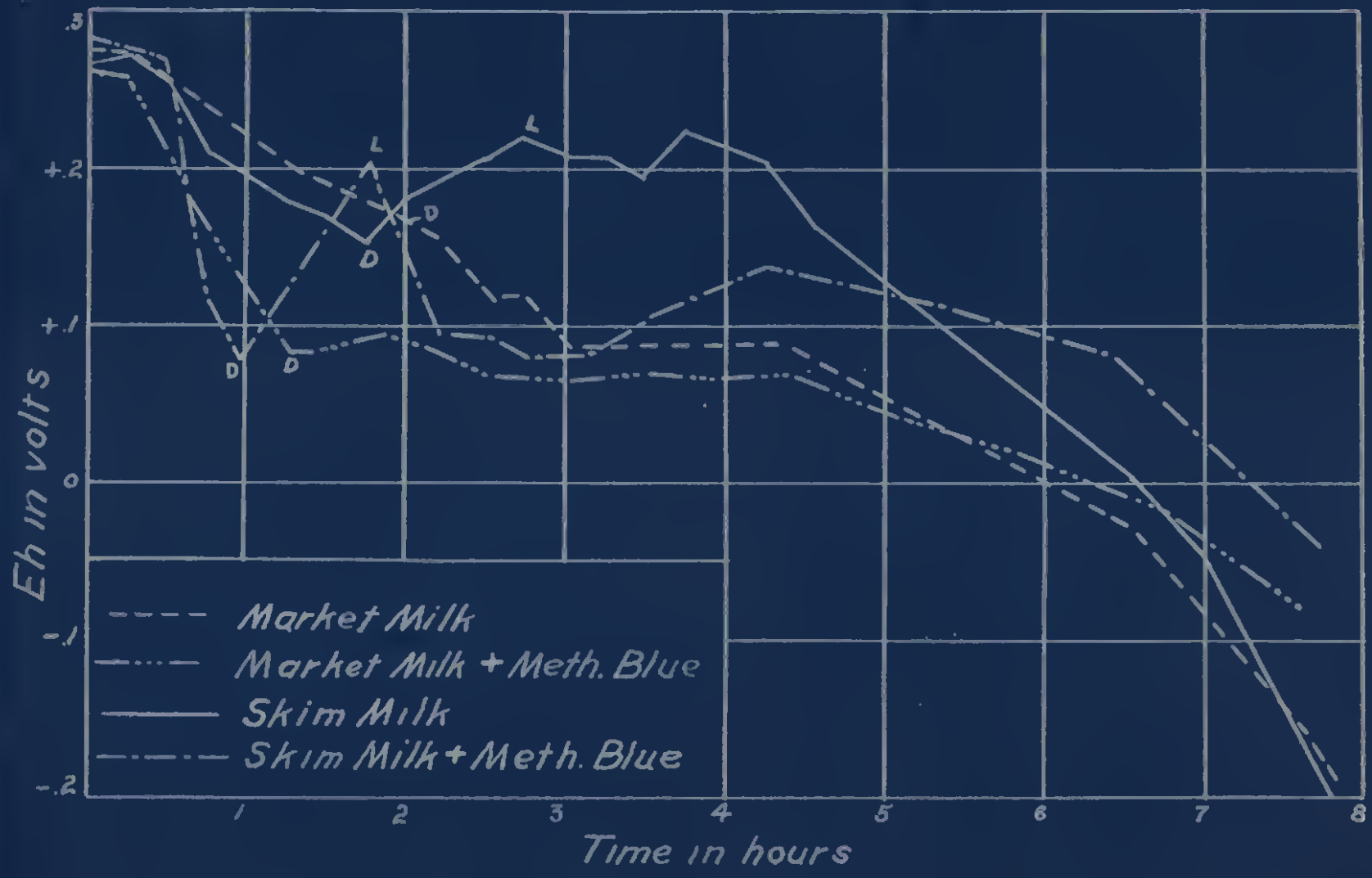

Fig. 12 The effect of light on the potential: time curves of market milk and skim milk with and without the addition of methylene blue

at 37°C. by submerging the tubes about three-fourths inch into a water bath held at 41°C. When the potential of a sample thus exposed had drifted toward more negative values, it was covered with a sleeve of heavy black paper to exclude the light. The effect on the potential of alternately placing milk or skim milk in the light and dark is well illustrated in Figure 12. At the points on the curves labeled "D" the samples were placed in the dark, and at points "L" they were again exposed to light. It will be noted that the potential of the sample of skim milk plus methylene blue dropped quickly to Eh +0.08volt at the beginning of the experiment; when placed in the dark the potential rapidly returned to the more positive Eh value of +0.2 volt. When again placed in the light the potential drifted quickly back to an Eh of approximately +0.1 volt. After three hours of incubation the sun, although still shining had disappeared behind adjacent buildings, thereby diminishing the intensity of the effective light. Skim milk containing methylene blue (1:200,000) was consistently found to be very responsive to any diminution of light intensity, as is evidenced by the slight drift in potential between the third and fourth hour of the experiment. The effectiveness of the sunlight was completely gone after the fourth hour of this particular experiment.

The potential curve of skim milk without methylene blue

shows that alternate placing of the sample in the light and in the dark affects the potential drift. However, the response of the electrode potential to light is not as great as in the case of skim milk plus dye.

The potential curves of market milk with and without dye show that the potentials drift to more negative values when exposed to sunlight. A more rapid drift of potentials of market milk (with and without dye) did not return to more positive Eh values when placed in the dark. It is interesting to note that the sample of market milk when placed in the dark, not only failed to respond by swinging to more positive values, but continued its uninterrupted negative potential drift.

These observations suggest:(1) that methylene blue accentuates the response of the electrode potential to the effects produced by the presence or absence of light, and (2) the presence of fat has a deterring influence on the potential drift as induced by light. This latter observation concurs with those made in connection with Figure 7 and Figure 8.

Effect of Artificial Light

It is a common practice in the determination of quality of milk by the methylene blue reduction test to incubate the samples in a constant temperature incubator. The tempera-

ture of such incubators is usually regulated by using artificial light as a source of heat. It has been observed that when samples were incubated in this manner, those nearest the light were reduced in the least time.

In a preliminary experiment, samples near the light reduced 2.5 hours earlier than samples shielded from the light. In order to determine the cause for this difference in reduction time the following experiment was conducted. A sample of market milk of good quality was divided into two parts and the standard amount of methylene blue added. The samples were placed in an incubator maintained at 37°C. and heated with two 75 watt bulbs, one of which burned constantly and the other was operated intermittently by the thermostat. Duplicate tubes of milk were protected from the light by sleeves of heavy black paper and two others were exposed to the rays from the bulbs. Representative potential curves and reduction times of two of these are presented in Figure 13.

An examination of these curves will show that artificial light affected the potentials and reduction time in much the same manner as was observed in the case of sunlight. The potential of the exposed sample drifted slowly toward the negative side, whereas that of the shielded sample remained fairly constant for six hours. As shown in Figure 13 the exposed sample was completely reduced 2.5 hours sooner than

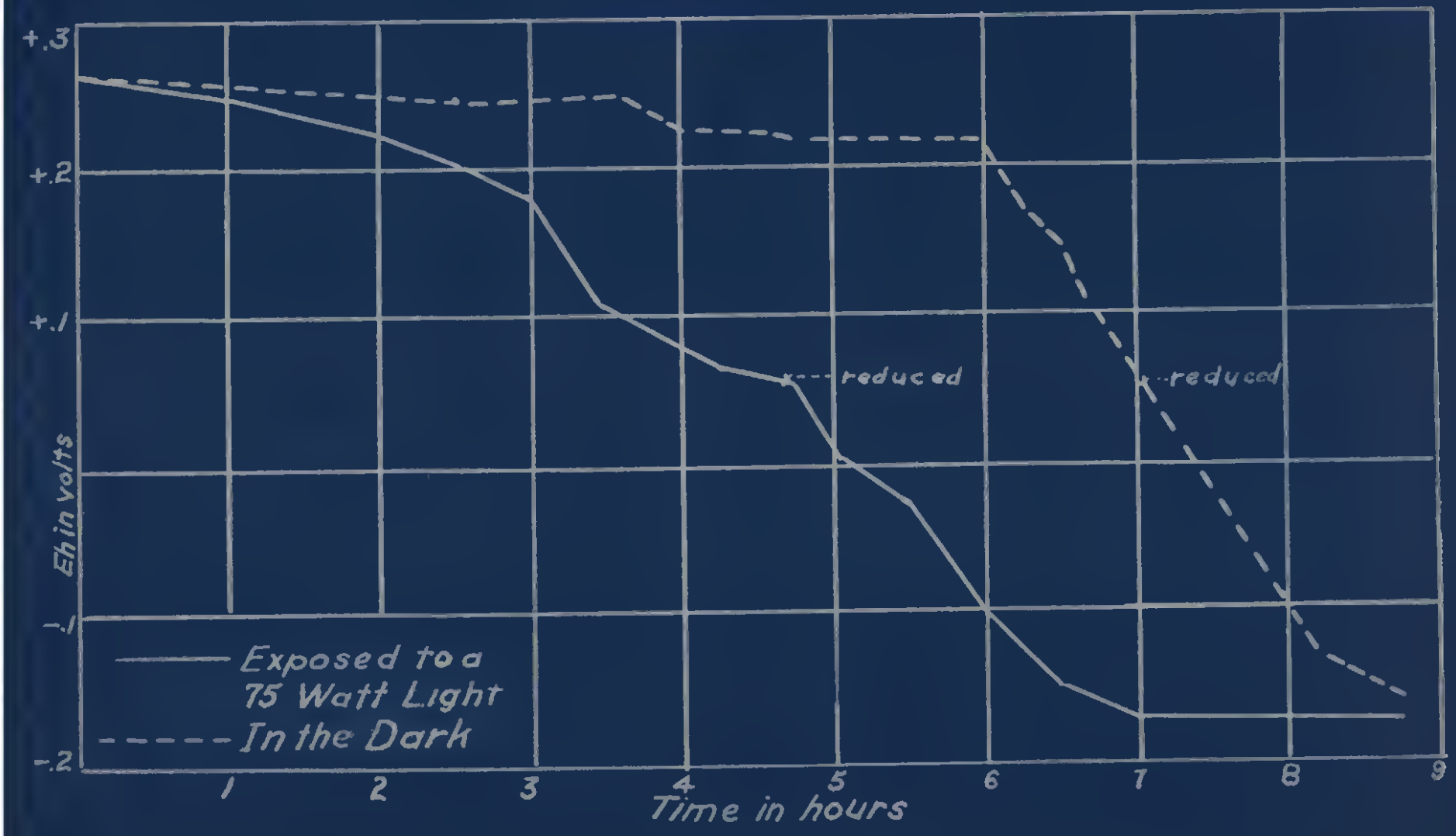

Fig. 13 Potential: time curves of a sample of market milk

was the shielded sample.

Temperature was not a factor in hastening reduction of the exposed samples. Temperature controls showed that the sample exposed to light was 1°C. lower than the one in the dark. Since the temperature of exposed sample was slightly lower than the one in the dark, it is untenable to attribute the more rapid reduction of this factor.

SUMMARY

The potential:time curves of milk with and without methylene blue remained in close agreement during the entire reduction process. The blue color and initial potentials of reduced samples could be restored by vigorous shaking or aspirating with air. Either of the above treatments also restored the initial potentials of samples without dye.

The position of the zone of visible reduction was caused to vary by altering either the fat content of the sample or the concentration of dye added. The zone became more positive with an increase in the percentage of fat and more negative with an increase in concentration of dye.

The time required for visible reduction increased as the zone of reduction became more negative.

When excessive amounts of dye (1:10,000) are added the potential of the solution does not pass smoothly to more negative limits, but is deterred as it approaches the zone

of reduction characteristic of this indicator.

The addition of cane sugar to cream delayed the potential drift and reduction time of the dye.

The potentials of cream, whole milk, and skim milk drifted toward the negative side when these solutions were exposed to sunlight. This potential drift was accentuated by the presence of smaller per cents of fat. Potential changes to both more positive and more negative values were deterred by the presence of fat. This influence exerted by fat was especially noticeable when solutions were alternately placed in sunlight and in the dark.

The addition of methylene blue to skim milk or cream accentuated the potential changes induced by sunlight. The reduction of skim milk by sunlight was hastened by the addition of sodium oleate or sodium stearate. With each increase up to 1:25,000 in the concentration of dye added to skim milk containing sodium oleate, the reduction intensities induced by sunlight were progressively more negative.

Since a variation in the amount of fat in a sample alters the potential of the zone of reduction, cream, whole milk, and skim milk have a different and characteristic zone. The potentials of the zone of reduction of whole milk and skim milk are fairly consistent, although the zone for cream varies with the fat content. Visible reduction induced either by sunlight or bacterial activity takes place within

the Eh limits characteristic for the particular sample.

The reducing intensity induced by bacterial activity is more negative than that induced by sunlight. In the case of sunlight the negative limits reached are seldom below zero as compared with a reducing intensity of -0.2 volt induced by bacteria.

These observations confirm Whitehead's (1930) conclusions, that reduction of methylene blue by light is a reaction, distinct from the reaction induced by bacteria.

It was observed that decolorization of methylene blue occurred whenever a negatively drifting potential passed through the zone of reduction of this dye. Similarly the blue color reappears when the potential is allowed to return to the requisite positive value. When skim milk plus methylene blue which had been reduced by sunlight was placed in the dark, the potentials quickly returned to sufficiently positive values to permit a return of the blue color.

Artificial light hastened the reduction of methylene blue in market milk. Light from a 75 watt electric lamp induced a potential drift in milk which differed only in degree from that observed in the case of sunlight. The reduction of methylene blue in one sample of milk was hastened 2.5 hours by exposure to light from an electric bulb.

CONCLUSIONS

(1) The zone of reduction becomes more negative with increasing concentrations of dye, thereby increasing the length of time required for reduction.

(2) The zone of reduction becomes progressively more positive with increasing percentages of fat.

(3) The reducing intensities induced by sunlight are not so great as those induced by bacterial activity.

(4) The inducing intensities induced by light are sufficiently negative to reduce methylene blue.

(5) The light emitted from a 75 watt Mazda lamp hastens reduction of methylene blue in milk.

(6) The presence of fat has a deterring influence on the potential drift as affected by light.

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