LXIV. THE REDUCTION OF METHYLENE BLUE IN MILK.

THE INFLUENCE OF LIGHT.

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THREE types of reaction which will bring about the reduction of methylene blue added to milk have been known for a considerable time and have been utilised in various ways.

(i) Schardinger [1902] has shown that milk contains an enzyme which is able to catalyse the reduction of methylene blue in the presence of an aldehyde. Trommsdorff [1909] has proved that the enzyme is a constituent of the milk itself, and is independent of micro-organisms. Other observers have shown that aldehydes may be replaced by xanthine, and methylene blue by nitrates. Hence the reaction is now recognised as a typical oxidation-reduction in which hydrogen donator and hydrogen acceptor are enabled to interact through the presence of an enzyme or catalyst.

(ii) Milk which has been heated for a short time to a temperature over 100° will bring about the decoloration of methylene blue. The mechanism of this reaction has not yet been definitely established. The possibility of enzyme action is of course excluded. Barthel [1925] considers that an oxidation-reduction occurs in which citrate acts as hydrogen donator, methylene blue as hydrogen acceptor, and some inorganic constituent of milk as catalyst. Schwartz [1929], however, has brought forward evidence in favour of the theory that hydrolytic products of the milk proteins, probably those containing sulphydryl groups, are the reducing agents.

(iii) Milk containing living bacteria will decolorise methylene blue. The reaction in this instance is caused by an enzyme associated with the micro-organisms, and most probably is an oxidation-reduction in which the methylene blue acts as a hydrogen acceptor. The corresponding oxidation, which occurs simultaneously, must be a reaction which supplies energy for the growth of the organisms. Clark, Cohen, and Gibbs [1928] have shown that a reaction takes place in the absence of methylene blue, and that the dye, if present only in small quantity, is merely an indicator of reducing potential, analogous to an indicator of hydrogen ion concentration. Milk has therefore no specific function in the reaction, but is merely the source of substances suitable for the supply of energy to bacteria.

The third reaction has been used for many years as a means of determining the degree of contamination of market milk supplies, and it was while applying it for this purpose that the observation was made that light has a significant effect on the progress of the decoloration of methylene blue in milk. Some samples of milk to which methylene blue had been added were incubated in a water-bath with glass sides, designed to facilitate observation of the tubes. All the samples gave unexpectedly short reduction times averaging 2 to 3 hours. On repeating the test with the same samples of milk, but using an ordinary metal water-bath, many of the samples took more than 7 hours to reduce. Sunlight evidently had influenced the progress of the decoloration.

So far as can be ascertained, there is no reference in the literature on methylene blue reduction in milk to any observation of the effect of light. Clark, Cohen and Gibbs [1928] have observed an oxidising effect of ultra-violet light on methylene white in aqueous solution, and Webster, Hill and Eidinow [1924] have used the decoloration of methylene blue in the presence of acetone for measuring the intensity of ultra-violet light. The latter reaction is said to be due to the decomposition of the acetone with the formation of reducing substances.

Experiments were devised in an attempt to find an explanation of the mechanism of the process in milk. It was necessary first to determine whether an enzyme of the milk or of the contaminating organisms was concerned in the reaction. Reductase tests were set up in duplicate with fresh milk and some of the same milk heated for half an hour in a boiling water-bath. One series was incubated in water at 37° in a thin-walled glass vessel exposed to bright sunlight, and the other series was incubated in darkness. The tubes which were not exposed to sunlight retained the blue colour for more than 7 hours. In the series exposed to sunlight there was very little difference between the heated and unheated milk samples. They showed decoloration in times which varied from half an hour to several hours, according to the intensity of the sunlight. Usually the heated milk samples were decolorised in rather shorter time than the corresponding unheated samples, but this was not always the case, and probably the difference was due to the loss of dissolved oxygen brought about by heating, and the varying degree of subsequent aeration. In every instance the lower part of the tube was the first to lose the blue colour and the decoloration progressed upwards. There was always a blue ring left at the top of the tube and on dull days the decoloration was never complete in the upper half of the milk. In particularly intense sunlight the decoloration proceeded until there was merely a blue ring at the milk surface. Thereafter the blue colour was slowly regenerated in the upper part of the tube, and sometimes reached halfway down the milk column after several hours. In some experiments a layer of liquid paraffin was superimposed on the milk to avoid any influence of atmospheric oxygen, but with heated milk no difference was observed. In unheated milk decoloration

occurred a little more rapidly when the milk was covered with paraffin, but the differences were hardly significant. This is in accord with the findings of Thornton and Hastings [1929] that diffusion of atmospheric oxygen into milk is much too slow to affect the reductase test. The number of bacteria in the milk seemed to have no bearing on the phenomenon in unheated milk, so long as the reduction caused by the organisms was sufficiently delayed, so that it was not superimposed on the light effect. In some experiments milk was obtained with sterile precautions directly from the cow into a sterile flask and the test was performed immediately. The results were exactly the same as those obtained with milk several hours old. This rules out the possibility that some heat-stable substance produced by bacterial action is necessary for the reaction.

It is evident therefore that the reaction takes place between some chemical constituent of the milk and methylene blue under the influence of sunlight, or that some reaction takes place in the milk itself which produces a reducing potential sufficient to reduce methylene blue. In the latter case the dye would be acting merely as an indicator of the oxidation-reduction potential; in the former case it would be essential to the progress of the reaction. Electrometric measurements of the oxidation-reduction potential in the presence and the absence of the dye will be necessary to decide which explanation is correct.

In a consideration of the substances in milk which may be concerned in the reaction, the milk-fat attracted attention, for it is well known that some of the unsaturated fats in cream undergo oxidation more readily in the presence of light. To determine whether the fat was responsible for the reducing action, milk was run through a centrifugal separator and reductase tests were performed on the original milk and on the separated milk. In bright sunlight the tubes containing separated milk suffered no decoloration, whereas the whole milk tubes became colourless as usual in a longer or shorter time, depending on the intensity of the light. The blue sometimes faded temporarily in separated milk, but returned in an hour or two to its full depth. A layer of liquid paraffin on the milk again had no effect, so that the greater ease of diffusion of oxygen into separated milk could not be the explanation of the absence of decoloration. The substance necessary for the reaction must be a constituent of the cream.

As a further test of the fat oxidation hypothesis some experiments were carried out in which salts of known fatty acids were added to separated milk in an endeavour to replace the substance which reacts with methylene blue. The sodium salts of palmitic and oleic acids were chosen as representatives of the saturated and unsaturated types. Clear-cut differences were immediately observed in reductase tests incorporating these substances and separated milk. The sodium palmitate had no effect within 7 hours, while the sodium oleate induced reduction in a short time in sunlight and had no effect in darkness. This seems strong evidence in favour of the theory that the

reduction of methylene blue in milk under the influence of sunlight is an oxidation-reduction phenomenon in which unsaturated fats are oxidised and methylene blue acts as hydrogen acceptor. The occasional return of colour in strong sunlight in the top layers of milk where fat is collecting may possibly be due to an autoxidation of the oxidised fats resulting in a final oxidation-reduction potential sufficient partly to re-oxidise the methylene blue. Further evidence would be needed on this point.

Further work is projected in which the phenomenon will be examined in a more quantitative manner with the aid of a quartz mercury-vapour lamp, for it seems most probable that the active agent is ultra-violet light and that more powerful effects will be observed than with sunlight acting through one or two layers of glass. The phenomenon has obvious practical importance in connection with the keeping qualities of butter-fat in the presence of hydrogen acceptors.

EXPERIMENTAL.

In all reductase tests 20 cc. of milk were measured into a 6" by $\frac{5}{8}$ " test-tube and 1 cc. of methylene blue solution was added. The dye solution was prepared from tablets supplied by Blauenfeldt and Tvede, Copenhagen, for the purpose of determining the bacterial reduction of methylene blue in market milk. The final concentration of dye in the milk was about 1 in 100,000. The tubes used had been previously sterilised and when the tubes were inverted to mix the contents, the operator's finger was washed with alcohol on each occasion. The samples were exposed to sunlight by immersing them in water at 37° in a thin beaker set close to the laboratory window.

The effect of sunlight on methylene blue reduction in heated and unheated whole milk.

Milk was measured into test-tubes some of which were immersed in a boiling water-bath for half an hour. The heated samples were thereafter cooled in water and inverted several times to aerate the milk to a moderate degree. Methylene blue was added to all the tubes and one set was incubated exposed to sunlight while a control set was incubated in darkness.

Table I. *Times of decoloration in hours.*

	In the light		In the dark	
	Unheated milk	Heated milk	Unheated milk	Heated milk
Bright day	2.25	1.75	All over 7	All over 7
Bright day	1.5	0.5	"	"
Dull day	{ 1.5 (only $\frac{1}{2}$ decol.) }	{ 1.5 (only $\frac{1}{2}$ decol.) }	"	"
Dull day	{ 4 (only $\frac{1}{2}$ decol.) }	{ 4 (only $\frac{1}{2}$ decol.) }	"	"
Bright day	1.5	1.75	"	"

The above results are typical of many observations. On dull days the decoloration usually progressed no farther when the lower half of the milk

column was colourless. The variation between unheated and heated milks is probably accounted for by different degrees of aeration. The time of decoloration may also vary with different milks.

The effect of sunlight on methylene blue reduction in whole and separated milk.

Milk was run through a sterilised centrifugal separator and both the original milk and the separated milk were used for reductase tests. Both milks were used in the heated and unheated states and in some tubes a layer of liquid paraffin was added. Bacteriological tests on the milk before and after separation showed that no measurable contamination had taken place.

Table II. *Times of decoloration in hours, in the light.*

	Whole milk	Whole milk + paraffin	Whole milk heated	Whole milk heated + paraffin	Separated milk	Separated milk + paraffin	Separated milk heated	Separated milk heated + paraffin
Bright day	2	0.5	0.5	0.5	>7	>7	>7	>7
Dull day	1.5	2	1.5	3.5	>7	>7	>7	>7
	(only $\frac{1}{2}$ decol.)	(only $\frac{1}{2}$ decol.)	(only $\frac{1}{2}$ decol.)	(only $\frac{1}{2}$ decol.)				
Dull day	4	4	4	4	>7	>7	>7	>7
	(only $\frac{1}{2}$ decol.)	(only $\frac{3}{4}$ decol.)	(only $\frac{1}{2}$ decol.)	(only $\frac{1}{2}$ decol.)				
Bright day	1	—	—	—	>7	—	—	—
Bright day	1.5	—	—	—	>7	—	—	—

All the controls incubated in darkness were not decolorised in 7 hours. The difference between whole milk and separated milk was always clear cut and it is evident that diffusion of atmospheric oxygen has no appreciable effect.

The effect of the addition of salts of fatty acids to separated milk.

Milk was freed from fat as previously described. Sodium oleate and sodium palmitate were added in amounts of approximately 0.5 g. to different tubes of whole and separated milk, and partially dissolved by gentle shaking. Reductase tests were set up as before.

Table III. *Times of decoloration in hours, in the light.*

	Whole milk	Separated milk	Whole milk + Na oleate	Whole milk + Na palmitate	Separated milk + Na oleate	Separated milk + Na palmitate
Bright day	1.75	>7	1	1.25	0.5	>7
Bright day	—	>7	—	—	1.5	>7

All the controls incubated in darkness were not decolorised in 7 hours. These are typical of several experiments on similar lines. There is thus strong evidence that an unsaturated acid such as oleic acid can replace some constituent of the cream in this reaction with methylene blue.

SUMMARY.

1. Methylene blue added to fresh milk of good quality is reduced in a short time in the presence of sunlight at 37°. In darkness at 37° no decoloration occurs within 7 hours.

2. The reaction in sunlight is not due to an enzyme, for it proceeds equally well in milk which has been heated to 100° for 30 minutes.

3. Milk from which the fat has been removed by centrifugal separation no longer gives the reaction, but the activity of the milk can be restored by an addition of sodium oleate. Sodium palmitate has not a similar action.

4. It is suggested that sunlight catalyses an oxidation-reduction reaction in which unsaturated fats are oxidised and methylene blue is reduced.

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