CLXXX. THE ACTION OF SUNLIGHT ON MILK

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SUNLIGHT has a specific and very interesting action on milk. Viale [1925], investigating the Schardinger reaction, observed that milk decolorizes methylene blue even without the addition of aldehyde if exposed to sunlight. Furthermore boiled milk retains this property although the aldehyde hydrogenase is destroyed, enzymic action being thus excluded.

Whitehead [1930] also studied this phenomenon and attributed it to an oxido-reduction in which the unsaturated fat acts as oxygen acceptor. This interesting hypothesis was not supported by further investigations. From Whitehead's experiments this explanation appears very probable. In fact, if the fat is removed, the milk no longer decolorizes methylene blue when exposed to sunlight. If a salt of an unsaturated fatty acid (e.g. sodium oleate) is now added, the phenomenon reappears. When the methylene blue is reduced, the milk acquires an unpleasant characteristic fishy odour. In 1928 Frazier also observed this phenomenon and attributed the unpleasant odour and flavour of the milk to the oxidation of the fat.

Whitehead attributed the changes undergone by the milk in his experiments to the action of ultraviolet radiations, being led to this suggestion by the fact that it has long been known that ultraviolet radiations catalyse the oxidation of unsaturated fats.

I have made a series of experiments to test this hypothesis. Samples of milk to which methylene blue had been added were exposed to ultraviolet radiation, either in open vessels, or, to avoid the oxidizing action of the ozone which is always formed, in closed vessels. No reduction of the methylene blue was ever observed, although the oxidation of unsaturated fats under the influence of ultraviolet radiation is known to occur. The reduction of the methylene blue must be due to the action of some other part of the solar spectrum.

Further investigations have proved that milk fat (or butter) is readily oxidized when exposed to solar radiation. The fat separated by centrifuging gives an intense Kreis reaction with phloroglucinol after a short exposure to sunlight. This oxidation occurs without the addition of methylene blue.

The oxidation of the fat, however, is not the only reaction which occurs on exposure of milk to sunlight and the reduction of methylene blue involves another mechanism. It is not excluded that correlation exists between the two phenomena, oxidation of fat and reduction of methylene blue, without one of them being actually conditioned by the other.

The following experiments were made to test Whitehead's theory:

Exp. 1. The fat from 50 ml. fresh milk, which strongly reduced methylene blue in sunlight, was separated by centrifuging, well washed with distilled water and dispersed in a phosphate buffer solution (*pH* 6.8). Methylene blue was added and the whole exposed to sunlight. The dye was not reduced even after a long exposure, whilst a strong fishy odour developed.

Exp. 2. The milk from which the fat had been separated no longer reduced methylene blue on simple exposure to sunlight, but when the exposure was made in an evacuated Thunberg tube, the methylene blue was completely reduced and the colour did not return even after a long exposure. When air was introduced into such an exposed tube, even in small amount, the solution immediately turned blue.

The following conclusions were drawn:

- (1) The fat does not take a direct part in the reduction of methylene blue.
- (2) Another hydrogen donor is present which reduces the methylene blue.
- (3) The unsaturated fat has a very important effect in fixing the dissolved oxygen of the milk. This fixation is favoured by the highly dispersed condition of the milk fat and is catalysed by sunlight. An anaerobic medium is thus produced in which the reduction of methylene blue can occur.

There are thus two phenomena concerned:

- (a) the oxidation of unsaturated fat under the influence of the sunlight, which is the cause of the fishy odour;
- (b) the transference of the hydrogen of a donor which produces the reduction of the methylene blue.

These two phenomena have not been distinguished by any of the workers who have hitherto studied the reduction of methylene blue by milk in sunlight. This is the reason why many of the hypotheses suggested are contradictory. Thus E. Martini [1933] studied the phenomenon electrometrically and concluded that the amino-acids of the milk played the chief part. He rejected the hypothesis of V. Martini [1931] who ascribed the reduction of methylene blue in sunlight to the action of reduced glutathione. Ravenna [1934] attributed the phenomenon to the fermentative action of bacteria stimulated by the solar radiation. Aikins & Fay [1932] maintain that the light lowers the oxido-reduction potential and that this promotes the reduction.

I have made the following experiments on this subject, endeavouring in the first place to clear up the part played by glutathione, since this compound is of great importance in many biochemical oxido-reductions.

The Gabbe method of estimation was used. 5 ml. of milk are deproteinized with 5 ml. 25% trichloroacetic acid, and to 5 ml. of the filtrate are added 1 ml. of a freshly prepared 25% solution of KI and 1 ml. of 0.01*N* iodine. After exactly 5 min. 2 drops of starch solution are added and the excess of iodine titrated with 0.004*N* $\text{Na}_2\text{S}_2\text{O}_3$. A blank test is made with 5 ml. of 10% trichloroacetic acid solution. 1 ml. of 0.004*N* $\text{Na}_2\text{S}_2\text{O}_3$ corresponds with 1.228 mg. reduced glutathione. The "glutathione" content of the milk of various mammals has been determined by this method (Table I), which, however, really determines "substances oxidizable by iodine" since it is not specific for glutathione.

Qualitative tests with sodium nitroprusside, even when the reaction was sensitized by the addition of ammonium sulphate or mineral salts, gave negative results for glutathione in all the milks examined except mare's milk. Attempts were also made to determine the glutathione by the specific method of Binet & Weller [1934; 1936], but no reduced glutathione was found. The results obtained by the iodine method are, however, for convenience, expressed in terms of "reduced glutathione".

The fat from a quantity of milk was separated by centrifuging and after being thoroughly washed was dispersed in a phosphate buffer solution, *pH* 6.8. Such a dispersion does not reduce methylene blue on exposure to sunlight. If reduced glutathione is added the mixture then reduces methylene blue on exposure.

Table I

Milk	"Glutathione reduced" mg./100 ml.	"Glutathione* total" mg./100 ml.
Woman (primipara)	0	195
"	36	180
"	0	122
"	36	163
"	35	184
"	17	158
Woman (secundipara)	0	48.7
"	0	69.7
"	0	52
Woman (tertiipara)	3.5	74.6
"	25.0	67.3
"	67.3	214.0
Cow	12.2	69.7
"	14.5	83.5
"	9.7	63.5
"	18.2	73.6
"	0	52.0
"	3.66	50
"	18.2	76
Buffalo	55.0	102
"	67	111.0
"	73.6	109.8
"	75	110
"	85.4	123
"	80	127
"	52	97.6
Sheep	61	111
"	65	122
"	73	109
"	55	110
"	73.5	130
"	80	163
"	61	135
Mare	140	395
"	150	427
"	130	360
"	138	372
"	122	361
"	165	404
"	140	380
"	122	366

* The determination of total glutathione was made by the method of Gabbe with potassium ferrocyanide [Rona, 1929].

Table II

Fat from milk of	Glutathione added mg.	Time of decoloration sec.
Cow	25	10
"	50	4
"	100	1
"	100	2
Buffalo	25	5
"	50	3
"	100	1
Sheep	25	2
"	50	1
"	100	Immediately
Mare	25	12
"	50	7
"	100	3

The influence of various quantities on the rate of decoloration is to be seen in Table II.

Hopkins [1925] has shown that glutathione favours the oxidation of unsaturated fatty acids of glycerides or lecithin esters.

The experiment described above proves that reduced glutathione in the presence of milk fat is able to reduce methylene blue, which is in agreement with V. Martini's hypothesis. This hypothesis, however, is disproved by the fact that milks which do not contain reduced glutathione nevertheless reduce methylene blue in sunlight. Determination of the substances oxidizable by iodine before and after exposure of the milk to sunlight (Table III) shows that in most cases the iodine-oxidizable substances are completely oxidized by the exposure.

Table III

Milk	Fat g./100 ml.	"Reduced glutathione" before exposure to sunlight mg./100 ml.	"Reduced glutathione" after exposure to sunlight mg./100 ml.	Time of decoloration sec.
Cow	3.5	12	0	6
"	3.3	15	0	5
"	4.0	3	0	10
"	2.0	0	0	∞
"	3.5	15	0	4
"	3.75	20	0	Immediately
Buffalo	7.0	56	0	1
"	6.0	60	0	1
"	6.5	32	0	2
"	8.5	34	0	2
"	11.0	80	10	Immediately
"	8.2	36	0	3
Mare	0.7	132	101	Immediately
"	0.8	140	120	1
"	1.0	125	110	1
"	0.5	111	90	1
"	0.9	123	100.5	1
Sheep	6.0	15	0	3
"	10.5	25	0	3
"	10.3	50	0	2
"	6.8	52	0	2
"	2.5	32	0	4
Woman	2.5	12	0	5
"	—	0	0	∞
"	—	31	0	2
"	—	68	0	Immediately
"	—	48	0	2
"	—	0	0	∞
"	—	0	0	∞

In these experiments, as in all to be described, 20 ml. milk were placed in a Petri dish 9 cm. in diameter and 5 drops of 1/2 methylene blue were added. The exposures were preferably made in May–August.

(The interesting fact was observed that in these cases the sodium thiosulphate required for the back-titration of the iodine was often in excess of the quantity required for the iodine added.)

The oxidation of the iodine-oxidizable substances in sunlight is independent of the fat content of the milk, since milk from which the fat had been removed gave approximately the same results (in vacuum) (Table IV).

It is seen from Table IV that the time required for decoloration in separated milk in vacuum is somewhat longer than in entire milk, but in all cases the

Table IV

Milk	Time of decoloration sec.	Time of decoloration in vacuum sec.	"Reduced glutathione" before exposure mg./100 ml.	"Reduced glutathione" after exposure mg./100 ml.
Cow: Entire	10	14	15	0
Separated	∞	30	25	0
Entire	12	16	13	0
Separated	∞	36	13	0
Entire	∞	6	2	0
Separated	∞	42	2	0
Buffalo: Entire	3	5	50	0
Separated	∞	8	50	0
Entire	Immediately	4	60	0
Separated	∞	10	60	0
Entire	3	7	50	0
Separated	∞	10	50	5
Sheep: Entire	1	2	60	0
Separated	830	14	60	10
Entire	Immediately	1	70	0
Separated	∞	5	70	0
Entire	2	3	55	3
Separated	∞	7	55	20
Woman: Entire	∞	150	1	0
Separated	∞	135	1	0
Entire	Immediately	2	60	0
Separated	∞	12	60	0
Entire	4	7	28	0
Separated	∞	10	28	5

methylene blue is completely reduced, whereas without evacuation reduction usually does not take place in the separated milk. This is in complete agreement with our view that the unsaturated fat of the milk rapidly combines, in sunlight, with the dissolved oxygen and thus creates anaerobic conditions in which reduction of the methylene blue occurs.

Since milk as a rule (p. 1453) does not contain reduced glutathione the question arises as to what is the oxidizable substance on which the reduction of methylene blue in sunlight depends. Booth & Kon [1934] have stated that milk after exposure to sunlight no longer reduces 2:6-dichlorophenolindophenol, which is known to oxidize ascorbic acid.

E. Martini & Bonsignore [1934] also attribute to sunlight the power to oxidize ascorbic acid and have even proposed a method of determination of ascorbic acid based on this property.

To ascertain the part played by ascorbic acid in the reduction of methylene blue by milk in sunlight, the following experiments were made.

Exp. 1. 20 ml. of milk were exposed to sunlight for 5 min. in the usual way. After this exposure the milk did not reduce methylene blue. After the addition of 0.1 ml. of a freshly prepared solution of 0.2% ascorbic acid,¹ the milk again rapidly reduced methylene blue on exposure.

¹ The ascorbic acid solution was made with "L-Ascorbinsäure", Schering Kahlbaum. The solution was always freshly made because ascorbic acid becomes oxidized spontaneously on standing. 1 ml. of 0.2% ascorbic acid solution:

On the first day required	...	2.67 ml. 0.004 N iodine
On the second day required	...	2.37 "
On the third day required	...	2.02 "

Exp. 2. The fat was removed from a quantity of milk by centrifuging, well washed with distilled water and dispersed in a buffer solution *pH* 6.8. This dispersion did not reduce methylene blue on exposure, but when 1 ml. of 0.2% ascorbic acid was added, reduction rapidly occurred on exposure.

Exp. 3. 5 ml. of 1% methylene blue solution are rapidly and permanently reduced by 0.1 ml. of 0.2% ascorbic acid solution. The reduction only occurs in sunlight. Exposure to ultraviolet radiation, however long, does not cause reduction of the methylene blue. A similar observation was made with milk, and this affords indirect evidence that the methylene blue reduction by milk is due to ascorbic acid.

Exp. 4. Varying quantities of ascorbic acid were added to milk and the substances oxidizable by iodine were determined before and after exposure (Table V).

Table V

mg. ascorbic acid added to 100 ml. milk	ml. 0.004 <i>N</i> iodine solution required before exposure	ml. 0.004 <i>N</i> iodine solution required after exposure
(1) 40	110	61
20	56	0
10	28	0
5	13.7	0
1	3	0
(2) 30	63	21.75
15	31.5	0
10	16.2	0
5	7.5	0
1	1.56	0

We see from Table V that in milk there is present only a limited quantity of substances which can be reduced by ascorbic acid in sunlight. The ascorbic acid becomes oxidized only in presence of a hydrogen acceptor. When this fails, the ascorbic acid remains unchanged.

Further evidence that the methylene blue reduction by milk exposed to sunlight is due to ascorbic acid is afforded by the fact that the reduction no longer occurs if the milk is treated with alkali (Table VI).

Table VI

Milk	"Reduced glutathione" before alkalization mg./100 ml.	"Reduced glutathione" after alkalization mg./100 ml.	Time of decoloration before alkalization sec.	Time of decoloration after alkalization sec.
Cow	12	0	10	∞
"	13	0	8	∞
Buffalo	56	8	3	500
"	50	10	Immediately	650
Sheep	50	4	6	∞
"	40	4	6	∞
Mare	132	12	2	200
"	128	15	7	178
Woman	35	0	5	∞
"	60	0	Immediately	∞

Naturally soured milk retains its power of decolorizing methylene blue in sunlight. Bacterial reduction does not interfere. The substances oxidizable by iodine also remain unchanged after natural acidification.

DISCUSSION

The experiments described prove that the action of sunlight produces two effects on milk.

(1) *Oxidation of unsaturated fat.* This phenomenon is independent of the decoloration of methylene blue. The reduction of methylene blue is, however, aided by this oxidation of the unsaturated fat, which produces anaerobic conditions in the milk by using up the dissolved oxygen and thus allows the second phenomenon to appear.

(2) *Oxidation by catalytic dehydrogenation of the ascorbic acid present in the milk.* This dehydrogenation is responsible for the decoloration of the methylene blue, which serves as hydrogen acceptor. When all the ascorbic acid has been oxidized, the colour of the methylene blue is restored if air or oxygen be admitted. The determination of the substances oxidizable by iodine before and after exposure to sunlight can be used to evaluate the vitamin C content of milk. The results obtained by this method agree well with those obtained by direct titration with 2:6-dichlorophenolindophenol by the method of Schlemmer *et al.* [1932]. The rate of reduction of methylene blue on exposure of the milk to sunlight does not give quantitative information about the content of ascorbic acid because this rate depends on the amount of unsaturated fat present, which plays the part described above (oxygen absorption).

With the exception of mare's milk, the milks examined did not contain reduced glutathione, as was proved by the negative reactions to the nitroprusside and cadmium lactate tests.

The properties of mare's milk are being further studied.

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