CCCXVII. THE EFFECT OF LIGHT ON THE VITAMIN C OF MILK.¹

BY STANISŁAW KAZIMIERZ KON AND MEARN'S BRUCE WATSON.

From the National Institute for Research in Dairying, University of Reading.

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IN estimating vitamin C chemically in milk by the method of Birch *et al.* [1933], Kon [1933] observed very marked fluctuations in the concentration of that vitamin from day to day.

The possible causes of this phenomenon were investigated by Mattick & Kon [1933], who ultimately found that milk which originally gave a positive vitamin C titration failed to reduce the indophenol reagent after a short exposure to light in glass bottles. Some time later Booth & Kon [1934] showed that 90 % of the original power of reducing the reagent could be restored after short exposures to light by treating the milk with hydrogen sulphide. Longer exposure entailed greater irreversible losses.

An example of the effect of light will be found in Table I.

Table I. *Vitamin C estimations carried out on milk from a pint bottle before and after half-an-hour's exposure to sunlight.*

Ascorbic acid in mg./100 ml. of milk.					
Before exposure		After exposure		Control wrapped in black paper and exposed	
Before treatment with H ₂ S	After treatment with H ₂ S	Before treatment with H ₂ S	After treatment with H ₂ S	Before treatment with H ₂ S	After treatment with H ₂ S
1.95	2.38	0.00	1.41	1.95	2.38

The titrations were carried out as described on p. 2275. The higher titration given by the unexposed milk after treatment with H₂S is most probably due to previous exposure to light in the dairy (see p. 2278).

The present paper deals with detailed investigations carried out since the preliminary papers were published.

EXPERIMENTAL METHODS.

Choice of analytical methods.

Booth & Kon [1934], when reducing the reversibly oxidized substance, treated the milk exposed to light with H₂S; the milk was then deproteinized with trichloroacetic acid and the serum freed from hydrogen sulphide and titrated.

When the method was applied to milk in which vitamin C had been destroyed by heating and aeration in the presence of copper a small residual titration was obtained in every case. As it was highly unlikely that any vitamin C could have survived the drastic treatment, estimations were also carried out by the mercuric acetate method of Emmerie & van Eekelen [1934]. The results

¹ Read in part before the Biochemical Society, June 12th, 1936 [Watson & Kon, 1936].

are set out in Table II. In all cases 0.1 ml. of a 3% solution of CuSO_4 was added to 100 ml. of milk. The milk was then heated to 90° and air was bubbled through for various lengths of time. It will be seen from the table that the method of Emmerie and van Eekelen indicates complete destruction.

Table II. *The destruction of vitamin C in milk by aeration at 90° in the presence of copper measured by the method of Booth & Kon [1934] and of Emmerie & van Eekelen [1934].*

No. of exp.	Time of heating hr.	Method of titration	Vitamin C content of milk in mg. per 100 ml.			
			Original milk		Heated milk	
			Before H_2S treatment	After H_2S treatment	Before H_2S treatment	After H_2S treatment
1	0.5	B. & K.	2.25	2.86	0.00	0.49
2	2.0	"	2.05	2.63	0.00	0.45
3	2.0	"	1.87	2.41	0.00	0.45
4	2.75	"	1.60	2.60	0.00	0.40
5	2.75	"	4.60*	5.50*	0.00*	0.44*
6	2.75	E. & v. E.	1.95	2.25	0.00	0.00

* About 3 mg. of ascorbic acid were added to this milk before heating.

On the other hand a residual titration (equivalent to some 0.4–0.5 mg. of ascorbic acid per 100 ml. of milk) is obtained in all cases in which the method of Booth & Kon [1934] is used. The residual titration is very constant and is independent of the time of heating and of the amount of ascorbic acid originally present in milk.

When the two methods are applied to normal milk the Emmerie & van Eekelen technique gives lower values and the difference is exactly the same as the residual titration obtained above (Table III). This makes it almost certain that this residual titration is due to substances other than ascorbic acid.

Table III. *Comparison of the mercuric acetate method (M.A.) with the trichloroacetic acid method involving reduction followed by deproteinization (T.A.R.) and with the trichloroacetic acid method involving deproteinization followed by reduction (T.A.D.).*

Method	mg. of ascorbic acid per 100 ml. of milk after reduction with H_2S				Difference, mg./100 ml.			
	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
M.A.	1.96	2.10	2.42	2.24	0.41	0.47	0.49	0.44
T.A.R.	2.37	2.57	2.91	2.68				
T.A.D.	—	2.10	2.42	2.24				

In the mercuric acetate (Emmerie & van Eekelen) method the H_2S treatment is applied not to milk but to serum after deproteinization.

When the trichloroacetic acid method used by Booth & Kon [1934] was so altered that deproteinization preceded reduction, results identical with those given by the mercuric acetate method were obtained. The residual titration is therefore no doubt due to the action of H_2S on milk with the formation of a substance which reacts with the indophenol reagent.

Ascorbic acid added to milk is recovered by the mercuric acetate method and by the modified trichloroacetic acid method quantitatively and with equal accuracy (Table IV). The error is well within the limits of accuracy of the methods.

Table IV. *Recovery of ascorbic acid added to milk.*

5.7 mg. of ascorbic acid added to 100 ml. of milk. Results in mg. ascorbic acid/100 ml. of milk.

Method	Ascorbic acid content of milk		Ascorbic acid		% error
	Original milk	Milk and ascorbic acid	Added	Recovered	
M.A.	2.24	7.83	5.7	5.6	-1.7
T.A.D.	2.24	7.83	5.7	5.6	-1.7

The following procedure was finally adopted for the estimation of ascorbic acid in milk.

Method and technique of estimation of vitamin C in milk.

In a later part of this paper (p. 2284) evidence is offered that on exposure of milk to light ascorbic acid undergoes a reversible oxidative change to a biologically active product, very probably dehydroascorbic acid. This can be again reduced by H_2S and reacts then with the indophenol reagent. It is further shown that the substance (or substances) which does not react with the reagent after reduction with H_2S is biologically inert. For the sake of simplicity the knowledge of these findings will be assumed from the beginning of the paper.

The titration before H_2S treatment is referred to as "reduced ascorbic acid"; that obtained after it, measuring the reduced and reversibly oxidized forms of vitamin C, is described as "total ascorbic acid". All values are expressed as mg. of ascorbic acid in 100 ml. of milk.

Indicator. An approximately 0.04% solution of 2:6-dichlorophenolindophenol in pre-boiled, glass-distilled, water. The indicator is standardized against lemon juice, which in turn is titrated with 0.01 *N* iodine according to the method of Bessey & King [1933].

Preparation of milk. To 25 ml. of milk 15 ml. of 20% trichloroacetic acid are added. The mixture is allowed to stand for a few minutes after shaking and is then filtered.

Estimation of reduced ascorbic acid. 5 ml. of clear filtrate are titrated from a microburette with the standardized indophenol reagent until a faint pink colour, which persists for 30 sec., is obtained.

Estimation of total ascorbic acid (reduced and reversibly oxidized). Through the remainder of the filtrate a moderate stream of H_2S is passed for about 10 min. The container is then stoppered and left overnight in the dark. On the following morning the serum, saturated with H_2S , which has become cloudy on standing, is filtered and the H_2S is removed by passing a rapid stream of oxygen-free nitrogen until the emergent gas fails to stain moist lead acetate paper. This takes on the average about half an hour. Titration is then carried out as above.

The nitrogen is purified as follows. Commercial nitrogen from a cylinder is passed through 4 wash-bottles containing alkaline pyrogallol and through a sulphuric acid tower followed by a trap. It then passes into an electrically heated combustion tube filled with copper turnings. The emerging nitrogen is led through a wash-bottle containing alkaline glucose and methylene blue¹ into the milk serum.

¹ A 1% solution of methylene blue is added drop by drop to a hot 1% solution of glucose in *N*/10 NaOH until a faint blue is obtained. The temperature is then raised until the solution becomes colourless.

INVESTIGATION OF THE EFFECT OF LIGHT ON THE ASCORBIC ACID OF MILK.

The work reported in the preliminary papers [Kon, 1933; Mattick & Kon, 1933; Booth & Kon, 1934] was carried out on milk in commercial pint bottles. In the present investigation it was found more convenient to expose milk in smaller quantities, usually 25 ml. For this purpose the milk was placed in 3 oz. sample bottles shaped like milk bottles, with walls 4 mm. thick. To speed up the reaction the milk was frequently exposed to direct sunshine. The milk was obtained either from individual Shorthorn cows or from pooled Shorthorn milk produced on the Institute's farm.

Effect of exposure to different sources of light and to different wave-lengths.

Exposure to artificial light. 25 ml. of milk were exposed for 2 hours at a distance of 1 ft. to the light of a 12 volt 48 watt motor-car head lamp bulb (Siemens, gas-filled) working at 10 V. and 2.8 A. The milk gave an original titration of 1.33 mg./100 ml. of reduced and 1.71 mg./100 ml. of total ascorbic acid and a titration, after exposure, of 0.37 and 0.59 mg./100 ml. respectively. A control sample protected from the light remained unchanged.

Exposure to the light of a mercury vapour lamp in glass and quartz. 25 ml. quantities of milk were exposed as above in glass and quartz for 0.25, 0.5 and 1 hour at a distance of 25 cm. to the unscreened radiations of a Kelvin, Bottomley and Baird mercury vapour lamp working at 106 V. and 2.5 amp. D.C. The results are given in Table V.

Table V. *Effect of exposure in glass and quartz to the light of a mercury vapour lamp on the vitamin C content of milk.*

Material	R. reduced, T. total ascorbic acid in mg./100 ml.							
	Original milk		Time of exposure					
	R.	T.	15 min.		30 min.		60 min.	
	R.	T.	R.	T.	R.	T.	R.	T.
Glass	2.32	2.64	1.28	1.76	1.12	1.60	0.32	1.04
Quartz			1.04	1.36	0.80	1.44	0.24	1.04

The figures show that a greater reversible change and greater destruction are brought about by exposure in quartz, especially in the case of shorter exposures. The difference is, roughly, some 20%. According to Coblenz *et al.* [1926] some 30% of the radiations of a mercury vapour lamp up to 600 $m\mu$ (the following paragraphs show that light beyond that wave-length is inactive) are of a wave-length shorter than 290 $m\mu$. As ordinary glass cuts off at about 310 $m\mu$, the loss due to passing through glass would be more than 30%. Shorter ultraviolet rays do not therefore appear to be any more potent than the longer radiations given off by the mercury vapour lamp. According to Ellis & Wells [1925], the visible radiations of the mercury vapour lamp are chiefly concentrated in two yellow-green lines at 576.4 and 579.1 $m\mu$ and a green line at 546.1 $m\mu$. In view of the experiments with a sodium vapour lamp reported below, it is doubtful whether the first two lines play any role in the reaction. The active rays which pass through glass may therefore be due to the green line, and/or to the strong lines in the near ultraviolet (at 365-365.4, 398.4 and 404.6-407.8 $m\mu$).

Exposure to daylight through transparent cellophanes of different colours. The relative destructive effects of different wave-lengths have been studied by exposing

25 ml. samples of milk in boiling-tubes of 25 mm. internal diameter covered with the following colours of cellophane (300 grade)—red, brown, lemon, blue and heliotrope. In addition milk in a clear glass tube and in a completely darkened tube was also exposed. Absorption spectra of most of the cellophane wrappings used in this investigation will be found in a paper by Davies [1934]. The samples were exposed for 1 hour to February daylight. The results are given in Fig. 1. It will be seen that red cellophane protected the vitamin C

Fig. 1. The ascorbic acid content of samples of milk after 1 hour's exposure to daylight passing through cellophane wrappers of different colours.

almost completely, whilst the greatest change was found in the tubes covered with blue or violet (heliotrope) cellophane.

Exposure to the light of a sodium lamp. The relative inefficiency of the longer wave-lengths in the destruction of vitamin C in milk, demonstrated by the previous experiment, has been further confirmed by exposing milk to the monochromatic radiations of a sodium vapour lamp (Osira, G.E.C.) working at 1.35 amp. and 14.4 V. Two 25 ml. samples were exposed, one for 1 hour at a distance of 1 ft., whilst the second was similarly exposed for 1 hour, and then for another hour at half the distance. The second sample received therefore the equivalent of 5 hours' exposure at a distance of 1 ft. As Table VI shows, the sodium vapour light was totally without effect on the ascorbic acid of milk.

Table VI. *Effect of monochromatic sodium vapour light on the ascorbic acid of milk.*

Exposed milk							
Original milk		Exposed milk				Control exposed in black wrappers	
R.	T.	A. Exposure for 1 hr. at 1 ft.		B. Exposure as A + 1 hr. at 6 in. = 5A.		R.	T.
1.79	2.27	R.	T.	T.	T.	1.79	2.27
		1.79	2.24	1.79	2.24		

It will be remembered that the car lamp bulb (p. 2276) working at 28 watts for 2 hours at a distance of 1 ft., caused the destruction of some 65% of the ascorbic acid originally present in milk. Compared with this the lack of effect of the sodium lamp working at 19 watts for the equivalent of 5 hours at 1 ft. shows that light of wave-length 589 m μ (and, doubtless, of longer wave-length) is inactive. The slight destruction caused by light filtered through "brown" or "lemon" cellophane is no doubt due to admixture of shorter wave-lengths. The experiments described above show that the shorter visible rays and the near ultraviolet are chiefly concerned with the vitamin C reaction. In the case of

daylight acting through glass the relatively much greater intensities available in the blue and violet regions of the spectrum would make them chiefly responsible for the light effect.

Penetration of the active rays of sunlight through milk. A thin layer of milk transmits an appreciable amount of light. The following experiment was carried out in order to see whether the transmitted rays are active. Milk was exposed to June sunlight in rectangular cells made of cleaned photographic plates placed one behind the other in a black box which was open at one end. The layer of milk in the cells was 7 mm. thick (Table VII).

Table VII. *Penetration of the active rays of June sunlight through milk.*

Time of exposure min.	Reduced ascorbic acid in mg./100 ml.			
	Original milk	1st cell	2nd cell	3rd cell
0	A. 2.06	—	—	—
	B. 2.26	—	—	—
15	—	A. 0.00	A. 1.99	—
30	—	A. 0.00	A. 1.92	—
60	—	A. 0.00	A. 1.62	—
	—	B. 0.00	B. 1.41	B. 2.23

Sufficient light passes through a 7 mm. thickness of milk to cause an appreciable drop in reduced ascorbic acid in the second cell. A layer of 14 mm., however, prevented action taking place in the course of an hour's exposure. It is noteworthy that, according to Leikola [1932], milk absorbs more strongly in the red than in the blue.

Effect of oxygen.

When the dissolved oxygen of milk is completely replaced by an inert gas the reaction does not take place. A container with milk was evacuated three times on a high vacuum oil pump and each time the vacuum was released in an atmosphere of oxygen-free nitrogen. The nitrogen was then bubbled through the milk for half-an-hour. The container was then stoppered and the milk exposed for 2 hours to afternoon February sunshine. The results are presented in Fig. 2.

Fig. 2. Effect of light on the ascorbic acid of milk in the presence of oxygen or of an inert gas. 2 hours' exposure to afternoon February sunshine.

Effect of previous heating of the milk.

Ascorbic acid is reversibly oxidized under the action of light just as readily in milk which had been pasteurized (30 min. at 63.5°) as in raw milk. For example, a 15 min. exposure to June sunshine brought the concentration of

reduced ascorbic acid in a sample of laboratory pasteurized milk from 2.10 mg./100 ml. to nil. The total ascorbic acid fell at the same time from 2.17 to 1.72 mg./100 ml. These results are very similar to data obtained under similar conditions for raw milk (Fig. 5 and p. 2280).

It would seem unlikely that the enzymes of milk, which are largely inactivated during pasteurization, play a role in the process of oxidation.

Comparison of the effects of light on whole and separated milk.

25 ml. samples of whole milk and of milk from which most of the fat had been removed by centrifuging were exposed for 25 min. to pale noon sunshine in early March. No difference was found between the samples in either the reduced ascorbic acid (which was completely oxidized during the exposure) or the total ascorbic acid. It should be observed that the amount of fat left in separated milk is quite large compared with the amount of ascorbic acid.

Effect of length of exposure; evidence of two separate reactions.

25 ml. quantities of milk were exposed to June skyshine. At the end of 1, 2, 5 and 8 hours analyses for vitamin C were carried out (Fig. 3). It will be seen that all the ascorbic acid originally present in the reduced form had undergone reversible oxidation within the first hour of exposure, and that irreversible

Fig. 3. Effect of length of exposure to light on the concentration of vitamin C in milk.

--- Total ascorbic acid.

— Reduced ascorbic acid.

changes took place progressively throughout the whole length of the experiment. It is at once apparent that two separate reactions must have taken place: firstly a reversible change of the ascorbic acid to a substance no longer able to bleach the indophenol reagent unless treated with hydrogen sulphide; to effect this first change both light and oxygen are essential and this reaction does not proceed in darkness (Tables XIII, XV and XVI); secondly a more gradual change of the product of the first reaction to a substance which cannot be caused by H_2S treatment to give a positive test with indophenol and is, as will be shown later, biologically inactive. The question arises whether or not light is essential to the second reaction as well.

Is light essential to the second reaction?

25 ml. samples of milk were exposed for half an hour to dull daylight (end of March). One sample and one control were titrated immediately after exposure and the remainder were placed in a dark cupboard and an exposed sample and a

control were removed and titrated after 1, 2, 3, 4 and 5 hours (Fig. 4). In the course of the half-hour exposure the reduced ascorbic acid has disappeared completely, leaving some 1.6 mg./100 ml. of the reversibly oxidized form. This has continued to change in the absence of light so that after a further 5 hours only 1/3, some 0.55 mg./100 ml., is left. The shape of the curve is very similar to

Fig. 4. Behaviour in darkness of the ascorbic acid of milk after reversible oxidation caused by exposure to light.

Fig. 5. Rate of decomposition of reversibly oxidized ascorbic acid after different periods of exposure to light.

— Exposed for 1 hour. - - - Exposed for $\frac{1}{4}$ hour.

that obtained in the course of continuous exposure to light (Fig. 3), and it is quite obvious that when only reversibly oxidized ascorbic acid is left the reaction proceeds spontaneously for a long time without the agency of light. The truth of this statement is confirmed by the experiments presented in Fig. 5. Five 25 ml. samples of milk were exposed to May sunlight for the shortest time necessary to cause the complete disappearance of the reduced form of ascorbic acid—in this case 15 min.—and 4 other samples were exposed 4 times as long. One sample of the first group was analysed immediately after exposure, another at the same time as the first sample of the second group. Thereafter samples

from both groups were analysed together at intervals. The graph shows that the shapes of the two curves were very similar and that almost identical end values were reached.

Investigation of the first reaction.

Order of reaction. In the experiments described so far the exposure to light had been mostly too long to allow of a measurement of the rate of formation of the reversibly oxidized form of ascorbic acid. As a rule the reduced ascorbic acid was found to have disappeared completely in the course of the exposure. For this reason estimations at frequent intervals were carried out and the rate of oxidation was followed. It was found that measurements made every 5 min. gave enough points for the calculation of the order of the reaction. Fig. 6 presents the results of two series of observations. The logarithm

Fig. 6. Order of reaction of the reversible oxidation by light of the ascorbic acid of milk.

Table VIII. *Reversible oxidation of the ascorbic acid of milk under the action of light.*

Concentration of the reduced form at 5 min. intervals and velocity constants of reaction.

Time min.	Reading mg./100 ml.		Difference		Velocity constant	
	1st series	2nd series	1st series	2nd series	1st series	2nd series
0	2.06	2.08				
5	1.44	1.56	0.62	0.52	0.0716	0.0575
10	1.03	1.18	0.41	0.38	0.0670	0.0558
15	0.74	0.89	0.29	0.29	0.0661	0.0564
20	0.25	0.67	0.49	0.22	0.2171	0.0568
25	0.00	0.45	0.25	0.22	—	0.0796
30	—	0.00	—	0.45	—	—

of the concentration of reduced ascorbic acid (mg. per litre) is plotted against the time of exposure and the straight lines obtained show unmistakably that the reaction is a unimolecular one. The bottom line was obtained in the shade

on the forenoon of a cloudless June day, the top one on a dull day similarly in June. For very low concentrations the points, as would be expected, are off the lines. The last point in the top series is well within the experimental error. The actual readings and the velocity constants calculated from the equation $k = \frac{2.303}{t} \log_{10} \frac{A_0}{A}$ (where A_0 and A are the initial and final concentrations in the time interval t) are given in Table VIII.

Effect of temperature. 25 ml. quantities of milk in darkened bottles were placed (1) in a refrigerator at 0°; (2) at room temperature, 21°; (3) in an incubator at 37° until the milks had attained the temperature of the surroundings. They were then exposed for 10 min. to skyshine (July) and titrations were carried out immediately afterwards (Table IX).

Table IX. *Effect of temperature on the reversible oxidation of the reduced ascorbic acid of milk under the action of light.*

Original milk	After 10 min. exposure to skyshine		
	0°	21°	37°
2.00	1.41	1.04	0.52
Velocity constant	0.0350	0.0654	0.1347

The temperature coefficient of the reaction, calculated according to Van't Hoff [1898] is 1.44 for the range of temperature 0–37°.

The second reaction.

Little can be said at present with regard to the order of the second reaction. Judging from the shape of the curves in Figs. 3, 4 and 5 there is little doubt that it is not unimolecular.

Fig. 5 shows clearly that the reaction does not proceed to completion. A similar conclusion will be reached after inspection of the data of Table X, in

Table X. *Decomposition of reversibly oxidized ascorbic acid in milk kept in darkness.*

Original milk Ascorbic acid		Concentration of reversibly oxidized (total) ascorbic acid after exposure to light (mg./100 ml.)									Control after 22 hr. Ascorbic acid	
		Immedi- ately after ex- posure	½ hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	22 hr.	Re- duced	Total	
Re- duced	Total											
2.20	2.42	1.99	—	1.03	0.88	0.74	0.59	—	0.59	2.13	2.36	
1.98	2.20	1.77	1.18	0.88	—	0.74	—	0.74	0.63	1.84	2.20	

which are shown the results of titrations of samples of milk which had been exposed for 15 min. to May sunshine—just long enough to convert all reduced ascorbic acid into the reversibly oxidized form. The samples were then kept in darkness and titrated at intervals.

Discussion.

The observations bearing on the effect of light on the ascorbic acid of milk may be considered from two points. In the first instance they may be compared with the behaviour of ascorbic acid itself in aqueous media. Secondly,

other changes which are known to take place in milk under the action of light can be considered.

As is known, ascorbic acid readily undergoes oxidation to dehydroascorbic acid which is unstable in aqueous solution and undergoes a spontaneous change by the addition of the elements of water and opening of the lactone ring to 2:3-diketo-1-gulonic acid. Dehydroascorbic acid itself can be converted quantitatively into ascorbic acid by reduction with hydrogen sulphide, but the diketogulonic acid cannot be reduced to more than a slight extent with this reagent [Herbert *et al.* 1933].

The close similarity of this chemical behaviour of ascorbic acid with the changes undergone under the action of light by the ascorbic acid of milk leaves little room for doubt that the first step of the actinic reaction consists in the formation of dehydroascorbic acid. Not only is the substance in milk obviously a product of oxidation, but it shares further with dehydroascorbic acid the properties of reverting to ascorbic acid when treated with H_2S and, as will be shown later, of being biologically active. As regards the further decomposition of the first oxidation product, it is possible, and even probable, that diketogulonic acid is spontaneously formed in milk. The evidence is only of negative character. The substance in milk, like the gulonic acid, is neither reduced by H_2S nor does it exhibit biological activity. While it is very likely that the two substances are identical it will be well to remember that a reaction in the complex environment of milk may take a very different course from that in a simple aqueous solution.

It has been known for some time (Davies [1936, 1, 2] has reviewed the literature on the subject) that changes of an oxidative character take place in milk under the action of light. The changes which occur when milk is exposed to sunlight in clear glass bottles are generally attributed to photochemical activation of the dissolved oxygen or oxygen diffusing in. Outwardly the action manifests itself in the tainting of milk which, owing to the oxidation of the fat acquires a "cardboard" flavour.

The effect of light on the vitamin C of milk is doubtless a part of the general oxidative changes induced in milk by photo-activation. Whereas the oxidation of the fat is of an autocatalytic nature, the reversible oxidation of vitamin C stops when the light is removed. It may be reasonably assumed that ascorbic acid is present in true solution and that the velocity of reaction between ascorbic acid and oxygen under the influence of light is relatively high. Ascorbic acid would thus have priority over the emulsified fat in the competition for the available oxygen. In any case, the unimolecular character of the curve of reversible oxidation of the ascorbic acid of milk indicates that a relatively large excess of oxygen is available for the purpose.

It has been shown in Table I that half an hour's exposure to sunlight is sufficient to change all the ascorbic acid in a pint bottle of milk into the reversibly oxidized form. On the other hand the experiments described on p. 2278 indicate that a layer of milk 14 mm. thick efficiently prevents any actinic action in the course of 1 hour's exposure to strong sunlight and that a layer of half that thickness cuts the activity down very considerably. The rapid spread of the reaction through 70 mm. of milk in a pint glass bottle must therefore be explained otherwise than by the penetration of the actinic rays into the milk. It is possible that a chain reaction is involved.

THE EFFECT OF LIGHT ON ASCORBIC ACID ADDED TO MILK.

So far in this paper the identity with ascorbic acid of the photo-labile substance in milk giving the indophenol reaction has been assumed, though not finally proved. The following experiments make the evidence in favour of this assumption, already very strong, virtually complete.

Synthetic ascorbic acid added to milk behaves on exposure to light in exactly the same way as the ascorbic acid originally present. Several examples of the effects of light on milk fortified by addition of ascorbic acid are shown in Table XI. A synthetic preparation (B.D.H.) was used.

Table XI. *Effect of light on synthetic ascorbic acid added to milk.*

R. reduced, T. total ascorbic acid in mg./100 ml.

No. of exp.	Original milk		mg. ascorbic acid added per 100 ml.	Milk + ascorbic acid		Titration after 1 hr. exposure		Titration after 2 hr. exposure	
	R.	T.		R.	T.	R.	T.	R.	T.
1*	2.14	2.49	About 4.5	6.89	7.14	0.00	3.10	0.00	2.59
						Titration after 3 hr. exposure		Titration after 7 hr. exposure	
2†	1.93	2.30	8	10.09	10.24	0.74	5.20	0.00	1.63
3†	1.86	2.15	8	9.95	10.17	0.00	3.34	0.00	0.89
4†	1.93	2.23	8	10.09	10.25	0.00	3.86	0.00	1.71

* 25 ml. samples exposed.

† 150 ml. samples exposed.

It was found that with the higher concentration of ascorbic acid longer exposures were necessary to obtain complete reversible oxidation. Exp. 2 (Table XI) shows, for example, that even after 3 hours' exposure, some ascorbic acid had been left in the reduced form. Obviously, owing to the longer exposures, variations in the intensity of light were much more likely to occur, and these account for the marked differences between separate runs. Otherwise the chemical behaviour of the added ascorbic acid leaves no reasonable doubt as to the identity with vitamin C of the substance in milk which gives the indophenol titration.

BIOLOGICAL TESTS OF THE EFFECT OF LIGHT ON THE VITAMIN C ACTIVITY OF MILK.

The results so far described do not solve the problem of the vitamin C potency of milk which has been exposed to light. For this reason biological tests have been carried out.

The planning of the biological tests presented certain difficulties. It was evident for example that milk after exposure to light would have to be fed and consumed at once to obtain a correct measure of the activity of the reversibly oxidized substance. Delay in consumption would bring about the further spontaneous changes which have been described in the preceding pages.

This implied feeding by pipette, but the relatively low concentration of ascorbic acid in milk would make it necessary to give some 50 ml. within a short time—again a serious difficulty. Finally, the work involved in exposing the milks to light and carrying out the titrations, made it desirable to shorten as far as possible the experimental period. It was decided to increase the concentration of ascorbic acid in milk, by addition of the synthetic substance and

thus cut down the volume of milk required for feeding. As for the method of testing, a combination of Harris & Ray's [1932] curative technique with an estimation of the ascorbic acid content of the adrenals [Svirbely & Szent-Györgyi, 1933] and of the liver was adopted. Macroscopical lesions noticed at autopsy were also taken into consideration. Non-pregnant female guinea-pigs were used for the test as well as males. The animals, weighing initially 250–300 g., were kept for 3 weeks on the scorbutic diet of Eddy [1929] and were then used for the tests.

It was originally hoped to feed three types of milk: milk containing ascorbic acid only in the reduced form, milk with a similar content of ascorbic acid but only in the reversibly oxidized form and finally milk giving no indophenol titration even after treatment with H_2S . In practice, owing to the long exposures to light required by milk containing added ascorbic acid (discussed in the preceding chapter) it was necessary to feed one level of reduced ascorbic acid and 2 lower levels of the reversibly oxidized form.

The treatment of the milk was as follows: the milk was assumed to contain 2 mg./100 ml. of ascorbic acid (final measurements were not completed till the following day). 8 mg. of synthetic B.D.H. ascorbic acid were then added per 100 ml. of milk, giving a fivefold increase in the concentration of ascorbic acid. A part of this milk was then fed by pipette without delay to guinea-pigs. Two

Table XII. *Biological tests of the effect of light on the ascorbic acid in milk.*

Treatment	Average daily intake of ascorbic acid (mg.)	Total ascorbic acid in adrenal (average)		Total ascorbic acid in liver (average)		Scurvy score	Average gain or loss in weight during experimental period g.
		mg./g.	mg. in organ	mg./g.	mg. in organ		
First experiment. Average daily intake of milk 15 ml. per guinea-pig. Duration of experiment 7 days.							
Unexposed milk; 3♀	1.42 (reduced form)	0.22	0.050	0.05	0.71	0	+ 1
Milk exposed 3 hr.; 3♀	0.61 (reversibly oxidized)	0.13	0.029	0.04	0.58	+(+)	- 12
Milk exposed 7 hr.; 3♀	0.30 (reversibly oxidized)	0.08	0.020	0.04	0.51	++(+)	- 23
Negative controls; 2♀	0.00	0.04	0.014	0.03	0.46	++++	- 58
Second experiment. Average daily intake of milk 13 ml. per guinea-pig. Duration of experiment 14 days.							
Unexposed milk; 4♂	1.30 (reduced form)	0.23	0.060	0.06	0.80	Trace (?)	+ 10
Milk exposed 3 hr.; 4♂	0.52 (reversibly oxidized)	0.10	0.023	0.04	0.80	++	+ 9
Milk exposed 7 hr.; 4♂	0.24 (reversibly oxidized)	0.07	0.018	0.03	0.42	++(+)	- 31
Ascorbic acid 1st level; 2♂	0.50 (reduced form)	0.12	0.032	0.05	0.96	+	+ 19
Ascorbic acid 2nd level; 1♂	1.00 (reduced form)	0.28	0.084	0.05	0.82	Trace	+ 34
Negative controls; 3♂	0.00	0.03	0.009	0.03	0.29	++++	-122

other samples were then exposed in clear glass bottles of 150 ml. capacity and internal diameter of 5 cm. on a window ledge facing south. The exposures lasted generally 3 and 7 hours. Examples of typical runs are given in Table XI. These milks were given after exposure to guinea-pigs in the same way as the unexposed milk. All feedings were done in a darkened room. Two separate feeding tests were carried out. In one test only female guinea-pigs were used and the milks were given for 7 days. The other test lasted a fortnight and employed only male guinea-pigs. In the second test control guinea-pigs receiving an aqueous solution of ascorbic acid were also included. The results are given in Table XII.

The results of the experiments show conclusively that exposure of milk to light entails a loss of the vitamin C activity and it appears that, while the reversibly oxidized form of ascorbic acid is biologically active, the product of the subsequent decomposition is inactive. When the results obtained in the second experiment with the milk exposed for 3 hours are compared with those given by 0.5 mg. of ascorbic acid the difference in activity between the reduced and reversibly oxidized forms does not seem to be marked. This is more in agreement with the findings of Hirst & Zilva [1933] than with those of Roe & Barnum [1936]. The method of titration of ascorbic acid in milk used in the present study appears to be a reliable guide to its vitamin C activity.

EFFECT OF EXPOSURE OF MILK TO LIGHT UNDER PRACTICAL CONDITIONS.

The following experiments were carried out in order to find out to what extent the vitamin C content of milk can be affected by exposure to light in the normal routine of handling sale milk as when e.g. it is delivered on the door-step.

Pint and half-pint bottles of sale milk from the Institute's dairy were exposed in sun or shade (end of May) for half-an-hour, 1 hour and 2 hours. They were then placed in darkness and analysed at intervals (Table XIII and Fig. 7). The degree of destruction varies materially with the intensity and the time of exposure, and to a less extent with the size of the bottle. The loss of vitamin C is quite noticeable, and an exposure for half an hour in the sun followed by half an hour in the dark will reduce to half the amount originally present in the bottle. Even after short exposure in the shade, standing for a few hours in darkness will reduce by half the vitamin C available in a bottle of milk.

RESISTANCE TO HEAT TREATMENT OF THE VITAMIN C OF MILK BEFORE AND AFTER EXPOSURE OF THE MILK TO LIGHT.

It is generally known that pasteurization diminishes the vitamin C content of milk. The loss is especially severe in the presence of copper from the pasteurizing plant or dairy utensils, which acts as a catalyst. But even in the absence of copper a varying proportion of the vitamin, depending on the method of pasteurization, is destroyed. As the ascorbic acid of milk, reversibly oxidized under the action of light, is in general less stable than the reduced form of the vitamin, it was of interest to investigate the behaviour of these two substances in the course of pasteurization.

Half-gallon amounts of milk were obtained from the dairy shortly after milking. They were placed in aluminium vessels and pasteurized in the laboratory by the holder method (30 min. at 145–150° F.). Samples were analysed before and after pasteurization. The heat treatment and sampling were carried out in the absence of light (Table XIV).

Table XIII. *Effect on the vitamin C content of sale milk of exposure in pint and half-pint bottles in sun and shade for varying lengths of time.*

No. of exp.	Length of exposure hr.	Type of exposure	Type of bottle	Original milk		Immediately after exposure		1 hr. after exposure		3 hr. after exposure		6 hr. after exposure		24 hr. after exposure		Control after 24 hr.	
				R.	T.	R.	T.	R.	T.	R.	T.	R.	T.	R.	T.	R.	T.
1	0.5	Sun	1 pt.			0.00	1.41	0.00	1.11	0.00	0.97	0.00	0.82	0.00	0.59		
		Shade				1.11	1.93	1.11	1.78	1.11	1.63	1.11	1.48	0.97	1.19		
	1.0	Sun		1.93	2.38	0.00	1.04	0.00	0.74	0.00	0.74	0.00	0.59	0.00	0.59	1.78	2.23
		Shade				0.85	1.71	0.82	1.56	0.82	1.45	0.82	1.34	0.82	1.11		
2	0.5	Sun	$\frac{1}{2}$ pt.			0.69	1.41	0.69	1.26	0.69	1.26	0.69	1.11	0.45	0.74		
		Shade				0.82	1.80	0.59	1.26	0.59	1.08	0.59	1.04	0.52	0.97	1.63	2.15
	1.0	Sun		1.82	2.30	0.59	1.63	0.48	1.19	0.56	1.08	0.56	0.97	0.37	0.97	1.71	2.23
		Shade				0.67	1.63	0.56	1.26	0.59	1.26	0.59	0.97	0.45	0.74	1.63	2.15
																1.71	2.23

R. reduced, T. total ascorbic acid in mg./100 ml.

Fig. 7. Effect of half an hour's exposure to light in sun and shade on the vitamin C content of sale milk in pint bottles.

Table XIV. *Effect of pasteurization on the reduced and reversibly oxidized ascorbic acid of milk.*

R. reduced, T. total, R.O. reversibly oxidized ascorbic acid in mg./100 ml.

No. of exp.	Raw milk		Pasteurized milk		% loss in pasteurization		Raw milk R.O.	Ascorbic acid de- stroyed in pasteuri- zation
	R.	T.	R.	T.	R.	T.		
1	1.72	2.10	1.57	1.72	8.7	18.1	0.38	0.38
2	1.95	2.25	1.87	1.95	4.1	13.3	0.30	0.30
3	1.87	2.10	1.65	1.65	11.8	21.4	0.23	0.45
4	1.78	2.04	1.78	1.78	0.0	12.7	0.26	0.26
5	1.65	2.10	1.57	1.83	4.8	12.9	0.45	0.27
6	1.80	2.25	1.72	1.80	4.4	20.0	0.45	0.45
7	1.87	2.02	1.76	1.80	5.9	10.9	0.15	0.22
8	1.78	2.08	1.78	1.78	0.0	14.4	0.30	0.30
Average	1.80	2.12	1.71	1.79	4.9	15.5	0.31	0.33

The table shows that the loss caused by pasteurization in the total (reduced and reversibly oxidized) ascorbic acid originally present in milk is more than 3 times greater than that suffered by the reduced form. This means that it is chiefly the reversibly oxidized acid that has been destroyed. In fact it can be seen from the last columns of Table XIV that the reversibly oxidized acid present in raw milk accounts quantitatively for the net loss due to pasteurization (that is for the difference in total ascorbic acid before and after the heat treatment).

It has been found that milk taken directly from the udders of normal cows contains only the reduced form of ascorbic acid. These findings will be presented in a separate paper but the effect of heat treatment on the ascorbic acid of such milk is given here. $2\frac{1}{2}$ pints of milk were obtained in the absence of light directly from the udder through the teat-cup lining of a milking machine into an aluminium pasteurizing vessel. The milk was pasteurized immediately at 145–150° F. for half-an-hour. Samples were taken in darkness for analysis before and after pasteurization. The results obtained with the milks of 2 cows are given in Table XV. These results show conclusively that reduced ascorbic acid is not affected by pasteurization provided that catalysing metals are absent.

Table XV. *The effect of pasteurization in the presence or absence of added copper on the vitamin C content of milk obtained in darkness.*

R. reduced, T. total ascorbic acid in mg./100 ml.

No. of exp.	Raw milk		Pasteurized milk, no copper added		Pasteurized milk, 7 parts per million of copper added as CuSO_4	
	R.	T.	R.	T.	R.	T.
1	2.17	2.17	2.10	2.17	—	—
2	2.02	2.02	2.02	2.02	0.00	0.45

For a final experiment 4 pint bottles of freshly produced milk were obtained from the dairy. 2 were kept in dark wrappers and 2 were exposed to intermittent July sunshine for 35 min., which was the minimum time necessary to produce almost complete reversible oxidation. The two types of milk were then pasteurized for half an hour in aluminium vessels. Table XVI confirms again the instability of the reversibly oxidized and the resistance to heat treatment of the reduced form of ascorbic acid.

Table XVI. *Effect of pasteurization before and after exposure to light on the ascorbic acid content of milk.*

R. reduced, T. total, R.O. reversibly oxidized ascorbic acid in mg./100 ml.					
Raw milk		Pasteurized milk		Raw milk R.O.	Ascorbic acid destroyed in pasteurization
R.	T.	R.	T.		
Milk not exposed to light.					
2.15	2.37	2.15	2.11	0.22	0.26
Milk exposed to light.					
0.26	1.63	0.22	0.52	1.37	1.11

It will be noticed that the figure for total ascorbic acid in unexposed pasteurized milk is lower than that for the reduced. The difference is however within the experimental error of the method.

All these experiments make it clear that it is primarily the amount of reversibly oxidized ascorbic acid present in milk that governs the loss of vitamin C during pasteurization by the holder method and not necessarily the presence or absence of oxygen during the process. By analogy with the mechanism of the change of pure ascorbic acid in aqueous solutions:

the thermal decomposition of the reversibly oxidized ascorbic acid in milk may well not require the presence of oxygen. This view is certainly in agreement with the experiments of Rundberg [1934], who found no difference between pasteurization at 63° for half an hour *in vacuo* or in contact with air, as far as the destruction of vitamin C was concerned.

Differences in the initial exposure of milk to light and therefore in the amount of reversibly oxidized ascorbic acid present in the milk probably account for the very divergent statements found in the literature as to the extent to which vitamin C is destroyed by pasteurization.

SUMMARY.

1. Milk giving a positive chemical test for vitamin C fails to reduce the indophenol reagent after exposure to daylight through glass; the reducing power can be restored to a varying extent by treatment with H₂S, but irreversible losses always take place.

2. Visible light of short wave-length (blue and violet) is mainly responsible for the reaction. Ultraviolet light is also probably active. Yellow and red are almost without effect.

3. The action of light does not take place in the absence of oxygen. The reversible oxidation under the action of light follows the laws of a unimolecular reaction and has a temperature coefficient of 1.4 for the range of temperature 0–37°. It is possibly a chain reaction. The decomposition of the reversibly oxidized ascorbic acid is of a more complicated order and does not run to completion.

4. It is suggested that the effect of light on the ascorbic acid of milk is a part of the general oxidative changes induced in milk by the actinic activation of oxygen. It is also suggested that the mechanism of the breakdown of ascorbic acid in milk is similar to the known oxidative decomposition of this substance, namely that dehydroascorbic acid is formed in the reversible oxida-

tion and that the opening of the lactone ring takes place in the course of the further irreversible changes.

5. Synthetic ascorbic acid added to milk behaves, under the action of light, in the same way as the ascorbic acid originally present.

6. Tests on guinea-pigs carried out on milks to which ascorbic acid had been added confirm the chemical findings. They show namely that the reversibly oxidized substance formed under the action of light is biologically active, but that the decomposition products which fail to decolorize the indophenol reagent after the treatment with H_2S are devoid of activity.

7. A pint bottle of milk exposed under practical conditions on the doorstep for half-an-hour in the sun and then kept for 1 hour in the darkness loses fully half of its original antiscorbutic properties.

8. Pasteurization by the holder method destroys the reversibly oxidized, but does not affect the reduced, form of ascorbic acid in milk. Milk as secreted by the normal cow contains only reduced ascorbic acid. The amount of destruction of vitamin C caused by pasteurization in the absence of catalytic metals depends on the previous exposure of the milk to light.

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