IX. CONDITIONS OF INACTIVATION OF THE ACCESSORY FOOD FACTORS.

By SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute.

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In some preliminary communications [Zilva, 1920, 1921] attention was drawn to the fact that the fat-soluble and the anti-scorbutic factors were inactivated on exposure to ozone in the case of the former and ozone and air in the case of the latter. It was further pointed out that the anti-scorbutic factor could withstand the temperature of 100° for two hours when air was excluded. These observations have found confirmation in the results obtained independently by other workers. Hopkins [1920] has shown that when air is bubbled through butter for four hours at 120° the fat-soluble factor becomes inactivated. Drummond and Coward [1920] have also demonstrated that the destruction of this factor takes place in butter-fat in presence of air rapidly at high temperatures and more slowly at lower temperatures. Hess [1920] and Hess and Unger [1921] found that milk incubated with hydrogen peroxide lost its anti-scorbutic properties, whilst orange juice, milk, and tomato juice treated with oxygen lost some of their anti-scorbutic potency. Dutcher, Harshaw, and Hall [1921] have since confirmed Hess and Unger's observations concerning the destruction of the anti-scorbutic factor by hydrogen peroxide and the writer's observation on the stability of this principle at high temperatures in the absence of air.

The object of this communication is to describe a series of experiments on the action of ozone and air on the three accessory factors, observations having been made at various temperatures. These experiments owe their origin to the fact that the inactivation of the fat-soluble factor in butter by exposure to a mercury-quartz lamp [Zilva, 1919] was found by the author to be due to the action of the ozone generated by the lamp and not to the action of the ultra-violet rays.

THE INFLUENCE OF OZONE AND AIR ON THE FAT-SOLUBLE FACTOR.

In the preliminary communication [Zilva, 1920] it was pointed out that when cod liver oil was exposed to the mercury-quartz lamp for 16 hours in the absence of air the activity of the oil was not destroyed by the action of the light. On the other hand six hours of exposure of the same oil to ozone in the dark entirely inactivated it.

In testing the above oils for the fat-soluble factor 2 g. of the oil were added to the daily basal diet of 20 g. when the rats had ceased to grow owing to the deficiency in the diet. The following are the results obtained:

*With the addition of oil exposed to the mercury-quartz lamp
in the absence of air.*

Rat I ♀ gained 30 g. in 17 days.

Rat II ♀ gained 24 g. in 17 days.

Rat III ♀ gained 39 g. in 17 days.

With the addition of oil exposed to ozone for six hours in the dark.

Rat I ♂. No gain in weight in four weeks. On addition at the end of that time of a similar dose of the unexposed oil the animal resumed growth and gained 37 g. in three weeks.

Rat II ♂. No gain in weight in four weeks. On addition at the end of that time of a similar dose of unexposed oil the animal resumed growth and gained 23 g. in 16 days.

Rat III ♂. Declined in weight and died 26 days after the commencement of the administration of the oil. Developed keratomalacia during the period of treatment with the oil.

Rat IV ♀. No gain in weight in four weeks. On addition at the end of that time of a similar dose of unexposed oil the animal resumed growth and gained 29 g. in three weeks.

From the above experiments it is quite evident that ultra-violet light as such has no deleterious action on the fat-soluble factor. On the other hand ozone does inactivate it. Moreover the action of ozone must be very drastic, when one bears in mind that cod-liver oil is very potent. The oil employed in the above experiments has been found capable of inducing growth in rats in daily doses of 1.7 mgm. The experimental rats on the exposed oil consumed 60–70 % of their diet, in other words 1.2–1.4 g. of the oil or about 750 times as much as the minimum dose necessary to induce growth, without showing any tendency to grow. Possibly a much shorter exposure would have also produced inactivation of this factor.

In the earlier part of this work whale oil was used instead of cod-liver oil. The former is much less active than the latter and was given up for that reason. At the time the experiments were carried out it was not definitely settled that the fat-soluble factor was associated with the unsaponifiable fraction of the oil and it was therefore considered advisable to study the change in the iodine value of the oil during exposure to the mercury-quartz lamp. The differences observed were of a low magnitude. The following are the figures of a representative experiment:

Iodine value of whale oil before exposure	...	...	119
Iodine value of whale oil exposed to the lamp for eight			
hours in the absence of air	...	...	117.6
Iodine value of whale oil exposed for eight hours in the			
presence of the generated ozone	...	...	114.5

The change is not significant and the results are analogous to those obtained by Hopkins [1920] with butter heated in a stream of air at 120°. No

change was recorded in the iodine value of the butter after the treatment. It is very doubtful whether a chemical change such as the iodine value of the oil can have any bearing on the accessory factor.

Some experiments in connection with the inactivation of the fat-soluble were carried out in connection with another inquiry. It was necessary to inactivate a large batch of very potent cod-liver oil and Hopkins' method was adopted for that purpose. This method has an advantage over the employment of ozone because it does not change the consistency of the oil to such a great extent. Air was aspirated through the oil heated at 120° for 18 hours. By this means a very active oil was inactivated to the extent that a daily dose of about 0.75 g. did not induce any growth in rats.

THE INFLUENCE OF OZONE ON THE WATER-SOLUBLE FACTOR.

Autolysed yeast juice was employed as a source for the water-soluble factor in this experiment. The juice was exposed to the ozone with constant shaking in the same apparatus and for the same time as the cod-liver oil. After the exposure the juice was very much altered in taste, smell and colour. It was incorporated in the basal diet free from the water-soluble factor after the rats had been on the restricted diet between three and four weeks. Three animals received 1, 2 and 3 cc. of autolysed yeast juice in their daily basal diet of 20 g. All the three animals responded by resuming growth, gaining 19, 24 and 15 g. respectively in 17 days. As 1 cc. of autolysed yeast juice is not much above the minimum dose capable of inducing growth there could not have been much destruction of the water-soluble factor in the juice by this very drastic method and we may conclude that this factor is decidedly more resistant to oxidation than the other two accessory factors. This resistance explains why on exposing autolysed yeast juice in a Petri dish to a mercury-quartz lamp no inactivation of the water-soluble factor takes place [Zilva, 1919]. Whether drastic oxidation inactivates the water-soluble factor at higher temperatures still remains to be ascertained.

THE INFLUENCE OF OZONE AND AIR ON THE ANTI-SCORBUTIC FACTOR.

The influence of ozone in the dark on the anti-scorbutic factor was next investigated. Decitrated lemon juice, namely lemon juice from which the organic acids are removed by neutralisation with calcium carbonate and which retains almost the entire anti-scorbutic potency of the original juice, was exposed to ozone in precisely the same way as the active oils and the autolysed yeast juice. Fresh batches were prepared every two or three days so that no deterioration could take place on storage. The treated juices, as well as the control untreated juices of the same batch, were administered to guinea-pigs which had been kept on a scorbutic diet of oats and bran and autoclaved milk (40 cc.) for 14 days. Daily doses of 3, 5 and 7 cc. were administered with the following results:

Guinea-pig I. Dose 3 cc. Commenced losing weight after three weeks. Declined gradually and died 44 days after the commencement of the experiment. At the post-mortem examination evidence of scurvy was found.

Guinea-pig II. Dose 5 cc. Declined and died 35 days after the commencement of the experiment. At the post-mortem examination evidence of scurvy was found.

Guinea-pig III. Dose 7 cc. Maintained its weight for 53 days. Chloroformed at the end of this period. At the post-mortem examination some evidence of scurvy was found.

A control dose of 3 cc. of the original decitrated juice was found to be adequate to prevent scurvy for 56 days; the animal was still growing when the experiment was discontinued.

One may conclude from the above experiments that the ozone inactivated the juice to a great extent. The writer has previously found [Zilva, 1919] that when decitrated lemon juice was exposed to the mercury-quartz lamp its anti-scorbutic potency was not destroyed or even impaired in spite of the generated ozone. This can be explained by the fact that the juice was exposed in a quartz tube and, unlike the butter in that experiment, was shielded almost entirely from the generated ozone. The anti-scorbutic, like the fat-soluble factor, is, therefore, inactivated by ozone but is not affected by ultra-violet rays.

In view of the results obtained in the above experiments it became of interest to ascertain whether less drastic oxidative treatment such as exposure to air would also inactivate the anti-scorbutic factor. Air was therefore aspirated through decitrated lemon juice for 12 hours. Batches of juice prepared every two or three days were employed. The following is the protocol of the experiment:

Guinea-pig I. Dose 3 cc. Just maintained its weight for 51 days gradually declining in health and showing symptoms of scurvy during this period. The animal was chloroformed owing to its distressed condition. At the post-mortem examination evidence of advanced scurvy was found.

Guinea-pig II. Dose 5 cc. Commenced declining in weight after 17 days. Chloroformed 48 days after the commencement of the experiment. At the post-mortem examination evidence of scurvy was found.

Guinea-pig III. Dose 7 cc. Gained 57 g. in 51 days. Chloroformed 51 days after the experiment commenced. Evidence of mild scurvy was found at the post-mortem examination in this case.

A control dose of 3 cc. of the original juice was found quite adequate to prevent scurvy for 51 days. The control animals were still growing when the experiment was discontinued.

This experiment affords evidence that the exposure of decitrated lemon juice to air for 12 hours diminishes its anti-scorbutic potency very appreciably.

The above observations suggested the possibility that the inactivation of the anti-scorbutic factor by heat may be due in part if not entirely to the

presence of air during the heating. Such a possibility was conjectured by Delf [1920] who found that orange juice and swede juice retained their anti-scorbutic potency to a marked extent after being heated for a considerable time at temperatures higher than 100°. In order to test this theory the following experiments were devised. Two lots of decitrated lemon juice were heated at 100° under a reflux condenser. One was heated for one hour during which time air was bubbled through. The second was heated for two hours, air being all the time rigorously kept out by bubbling carbon dioxide gas through the solution. In this case also fresh batches of decitrated lemon juice were employed every two or three days. The following results were recorded:

Decitrated lemon juice heated in air for one hour.

Guinea-pig I. Dose 1.5 cc. Commenced declining in weight after a month. Chloroformed 43 days after the commencement of the experiment. Evidence of scurvy was found at the post-mortem examination.

Guinea-pig II. Dose 4 cc. Commenced to decline after three weeks and died 41 days after the commencement of the experiment. Evidence of acute scurvy was found at the post-mortem examination.

Guinea-pig III. Dose 6 cc. Maintained weight for 36 days and then suddenly declined. Died 43 days after the commencement of the experiment. Evidence of scurvy was found at the post-mortem examination.

The same juice heated in an atmosphere of carbon dioxide for two hours.

Guinea-pig I. Dose 1.5 cc. Gained 122 g. in 74 days. Chloroformed. No evidence of scurvy was found at the post-mortem examination.

Guinea-pig II. Dose 4 cc. Gained 48 g. in 74 days. Chloroformed. No evidence of scurvy was found at the post-mortem examination.

Guinea-pig III. Dose 6 cc. Gained 138 g. in 74 days. Chloroformed. No evidence of scurvy was found at the post-mortem examination.

This experiment affords proof that the anti-scorbutic factor is quite stable when heated in the absence of air.

Decitrated lemon juice contains a considerable quantity of reducing sugar. On hydrolysis, however, the reducing capacity of the juice increases slightly, a reducing substance, most probably a sugar, being liberated. An experiment was devised to ascertain whether the anti-scorbutic potency is destroyed when the juice is hydrolysed under anaerobic conditions. Decitrated lemon juice was therefore hydrolysed on a water-bath under a reflux condenser for five hours with 2 N hydrochloric acid. The air was displaced during hydrolysis by carbon dioxide. After hydrolysis the solution was neutralised with sodium carbonate and tested out for its anti-scorbutic potency on guinea-pigs. A fresh preparation of lemon juice was hydrolysed every day for feeding purposes. The following results were obtained:

Guinea-pig I. Dose 1.5 cc. Declined after three weeks and died 32 days

after the commencement of the experiment. Evidence of scurvy at the post-mortem examination.

Guinea-pig II. Dose 1.5 cc. Declined after 23 days. Died 30 days after the commencement of the experiment. Evidence of scurvy at the post-mortem examination.

Guinea-pig III. Dose 1.5 cc. Declined after three weeks and died 26 days after the commencement of the experiment. Evidence of scurvy at the post-mortem examination.

Guinea-pig IV. Dose 3 cc. Gained 30 g. in 37 days. Chloroformed. At the post-mortem examination the animal was found in excellent condition, but showed evidence of extremely mild scurvy.

Guinea-pig V. Dose 3 cc. Gained 28 g. in 37 days. Chloroformed. At the post-mortem examination the animal was found in excellent condition showing no evidence of scurvy.

Guinea-pig VI. Dose 3 cc. Gained 16 g. in 37 days. Chloroformed. At the post-mortem examination the animal was found in excellent condition showing no evidence of scurvy.

Guinea-pig VII. Dose 5 cc. Gained 18 g. in 37 days. Chloroformed. At the post-mortem examination the animal was found in excellent condition showing no evidence of scurvy.

Guinea-pig VIII. Dose 5 cc. Gained 71 g. in 37 days. Chloroformed. At the post-mortem examination the animal was found in excellent condition showing no evidence of scurvy.

Guinea-pig IX. Dose 5 cc. Gained 30 g. in 37 days. Chloroformed. At the post-mortem examination the animal was found in excellent condition showing no evidence of scurvy.

No control was tested out with this experiment owing to some mishap, but numerous experiments performed by the writer with decitrated lemon juice show that the minimum dose capable of preventing scurvy in guinea-pigs is about 1.5 cc. The conclusion one can draw from this experiment is that the hydrolysis diminishes the activity of the juice, but in spite of the very drastic treatment very significant activity is retained. This further confirms the view that the anti-scorbutic factor is stable towards heat providing anaerobic conditions are observed.

SUMMARY.

1. The fat-soluble factor was destroyed in cod-liver oil by exposing it to ozone in the dark at ordinary temperature and by bubbling air through it at 120°.
2. The exposure of autolysed yeast juice to ozone in the dark did not destroy the activity of its water-soluble factor to any appreciable extent.
3. The exposure of decitrated lemon juice to ozone destroyed its anti-scorbutic activity. This was also destroyed by passing air through the solution at ordinary temperature.

4. Ultra-violet rays had no deleterious action on the accessory food factors in the absence of air.

5. Two hours' boiling did not destroy the anti-scorbutic potency of decitrated lemon juice in an atmosphere of carbon dioxide, whereas one hour's boiling in the presence of air destroyed its potency almost entirely.

6. Hydrolysis of decitrated lemon juice with hydrochloric acid for five hours impaired the anti-scorbutic activity of the juice. The juice however retained its activity to a very considerable extent.

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