CCIV. THE ACTION OF VITAMIN C ON THE OXIDATION OF TISSUES *IN VITRO*.

By DOUGLAS CREESE HARRISON.

From the Department of Pharmacology, Sheffield University.

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A FEW years ago, Szent-Györgyi [1928] isolated from the adrenal cortex a strongly reducing substance, "hexuronic acid." He showed that this substance could be reversibly oxidised and reduced, and suggested that it might play the part of an intermediary hydrogen carrier in tissue respiration. Recently, Svirbely and Szent-Györgyi [1932] produced strong evidence that "hexuronic acid"—since renamed ascorbic acid—is identical with vitamin C, and subsequent work has largely confirmed this.

It seemed probable that if vitamin C does play a part in tissue respiration, it might be possible to demonstrate its action by adding ascorbic acid to tissues which had been depleted of their store of vitamin C, that is, to the tissues of animals suffering from scurvy, and testing whether the addition of the vitamin increased the surviving respiration of the tissue. On carrying out the experiment, using the tissues of scorbutic guinea-pigs, it was found that the addition of vitamin C even in very small quantities does bring about a definite increase in the respiration of the tissues *in vitro*.

EXPERIMENTAL.

Guinea-pigs weighing about 300 g. were fed on a scurvy-producing diet consisting of bran, 60 %; rolled oats, 30 %; separated milk powder (heated for 2 hours in the oven at 120° with occasional stirring), 10 %. Water was given *ad lib.* and each animal received 0.5 cc. cod-liver oil weekly. The normal (non-scorbutic) control animals received the above diet with the addition of 3 to 5 cc. of fresh lemon juice daily. On this diet, the animals without lemon juice began to lose weight in about 3 weeks and showed definite symptoms of scurvy, while the control animals continued to grow. The animals were usually taken for experiment after about 3 weeks on the diet, though some were taken earlier than this. Equal numbers of male and female animals were used. The tissues examined were liver and muscle.

The guinea-pig was killed by a blow on the neck and allowed to bleed, and the tissues were quickly taken out and prepared, the liver by cutting thin slices by hand with a razor, and the leg muscle by cutting into small pieces with sharp scissors. Samples of the tissue were then weighed out on the torsion balance and suspended in phosphate buffer at p_H 7.4, either with or without vitamin C, in the bottles of the Barcroft differential manometric apparatus. Phosphate buffer was used in the experiments rather than a bicarbonate-Ringer solution, since it was desired to follow the course of the reaction, and in the manometric methods involving the use of bicarbonate buffer in equilibrium with a CO_2 gas mixture, it is only possible to take one final reading of oxygen uptake. The phosphate buffer

was prepared by treating $M/5$ KH_2PO_4 solution with the requisite amount of $N/5$ NaOH and diluting to 4 times its original volume. In the earlier experiments, the liver slices were cut with a razor moistened with buffer solution and were then washed in phosphate buffer, blotted on filter-paper and weighed. It was found, however, that much better agreement between duplicates was obtained if the liver slices were cut with a razor moistened only by the liver tissue fluid itself, the unwashed slices then being well mixed on a watch glass, divided into approximately equal portions with sharp scissors and equal amounts weighed out on the torsion balance. This technique was used in the majority of the experiments described in this paper, and the agreement between duplicate experiments on the same tissue was usually within 5 %.

In parenthesis, it may be mentioned that a very convenient method of checking the constants of the manometric apparatus was found to be given by the oxidation of alkaline ferrous sulphate. A suitable amount (depending on the range of the apparatus) of standard ferrous sulphate solution, slightly acidified with H_2SO_4 , was run into each right-hand bottle from a micro-burette, and, after bringing the apparatus into equilibrium in the bath and closing the taps, excess of NaOH was tipped into the solution. This can readily be done from a small tube suspended on the soda tube of the bottle. The oxygen uptake due to the oxidation of the ferrous iron reaches completion in about 15 minutes, and the method has the advantage that the calibration can be done under the same conditions as those under which the actual experiments are carried out.

The manometers used for the controls without vitamin C and the experiments with vitamin C were frequently interchanged.

Two different samples of ascorbic acid were used in these experiments. Both were colourless, crystalline preparations and, when titrated with iodine, took up exactly 2 atoms of iodine per molecule. The ascorbic acid solution was prepared freshly just before each experiment by dissolving the crystalline acid in a small volume of phosphate buffer and bringing the p_{H} back to 7.4 by adding the theoretical amount of $N/5$ NaOH . An equal amount of ascorbic acid in phosphate buffer but without tissue was always added to the left-hand bottle of the apparatus to compensate for the small oxygen uptake due to the autoxidation of ascorbic acid itself. In all experiments, the total volume of fluid in each bottle was 3.0 cc. The bottles were filled with oxygen rather than with air in order to reduce the possibility that the thickness of the tissue might be a limiting factor in the rate of diffusion and uptake of oxygen. A further reason for using oxygen was that the properties of ascorbic acid suggested that it might function as an intermediary hydrogen carrier between the reducing systems of the cell and activated oxygen. If this were so, its catalytic effect might be expected to be greater if the oxygen-activating systems of the cell were saturated with oxygen.

In filling with oxygen, the technique of Dixon and Tunnicliffe [1923] was used, the vessels being evacuated simultaneously to a pressure of about 100 mm. Hg and allowed to fill with oxygen, the evacuation and filling being carried out three times. The manometers were then shaken in a water-bath at 37° , allowing them to shake in the bath for 10 minutes before closing the taps and commencing to take readings. The CO_2 given off by the tissue was absorbed by rolls of starch-free filter-paper soaked in 40 % KOH in the small inset tubes in the bottles, and the oxygen uptake was measured over a period of 2 hours. About 45 minutes usually elapsed between the killing of the animal and the first reading on the manometer. Most of the determinations were carried out in duplicate on the same tissue both with and without vitamin C, and the mean values of oxygen uptake of the pairs with and without the vitamin respectively were compared.

Table I shows the effect of 0.25 mg. of ascorbic acid (concentration 0.08 mg. per cc.) on the oxygen uptake of liver slices from scorbutic guinea-pigs. The oxygen uptake figures are the totals over a period of 2 hours, and the tissue weights are given as wet weights. All the experiments, with the exception of the first three in the Table, were carried out in duplicate, both with and without addition of the vitamin, a total of 17 scorbutic guinea-pigs being used.

Table I. *Oxygen uptake of scorbutic liver respectively with and without 0.25 mg. vitamin C.*

Tissue weight in mg.	Alone mm. ³ O ₂	+ Vitamin C mm. ³ O ₂	Difference	Percentage increase
154	194	232	38	+20
174	252	272	20	+8
237	130	205	75	+57
300	466	541	75	+16
300	527	566	39	+8
285	286	370	84	+29
300	400	503	103	+26
225	144	164	20	+14
300	361	547	186	+52
185	145	173	28	+19
100	158	183	25	+16
255	523	600	77	+15
250	357	404	47	+13
152	338	373	35	+10
300	437	458	21	+5
207	405	450	45	+11
200	230	349	119	+52

It can be seen that there is a variable but very definite increase in the oxygen uptake of scorbutic guinea-pig liver produced by the addition of 0.08 mg. per cc. of vitamin C.

A few experiments have been carried out with 0.1 mg. ascorbic acid in 3 cc. and a definite increase in the oxygen uptake of scorbutic liver was produced. In one experiment with 0.05 mg. of the vitamin in 3 cc., a completely negative result was obtained.

Table II shows the effect of addition of 0.25 mg. of ascorbic acid in 3 cc. on the oxygen uptake of liver slices from nine normal guinea-pigs. Apart from the first two experiments in the table, the experiments were all carried out in duplicate and the conditions were identical with those in the experiments with scorbutic liver.

Table II. *Oxygen uptake of normal liver with and without 0.25 mg. vitamin C.*

Tissue weight in mg.	Alone mm. ³ O ₂	+ Vitamin C mm. ³ O ₂	Difference	Percentage difference
300	793	762	-31	-4
215	625	619	-6	-1
*197	91	95	+4	+4
214	416	367	-49	-12
200	467	466	-1	0
232	400	384	-16	-4
200	328	328	0	0
300	720	659	-61	-8
283	464	459	-5	-1

* In this experiment, the tissue had stood in air for 2 hours before testing.

It is clear that no increase is produced by addition of the vitamin to liver slices from normal guinea-pigs, in fact, if anything, there is a slight decrease in the oxidation in presence of ascorbic acid.

It should be mentioned that the results both in this table and those with scorbutic animals in Table I are in no way selected. They are the results of all the experiments I have carried out with the exception of one which was carried out on a guinea-pig obtained from another laboratory and believed to be suffering from scurvy. In this case, no increase in oxygen uptake was obtained on adding 0.25 mg. ascorbic acid to the liver slices, but on *post mortem* examination no signs of scurvy could be found in the animal.

It was not found possible to correlate the amount of increase in oxygen uptake produced on addition of the vitamin with the degree of scurvy from which the animal was suffering. While most of the scorbutic guinea-pigs showed definite haemorrhages around the knee joint, some of the biggest increases in oxygen uptake were obtained with animals which had been fed for a relatively short time on the scorbutic diet and which, though showing symptoms of scurvy on *post mortem* examination, appeared lively and well nourished. It appears very unlikely, therefore, that the effects observed were due to the inanition which occurs in the later stages of scurvy.

It was observed that in most of the experiments with scorbutic tissue, the accelerating effect of vitamin C on the oxygen uptake became much greater during the second hour than it was during the first hour, and the percentage increase in uptake produced by the vitamin calculated on the second hour alone gave much larger figures than those shown in Table I. Possibly this may be due to the slow diffusion of the vitamin into the tissue, or to a small residual quantity of vitamin C in the scorbutic tissues which becomes used up, the effect of added vitamin C then becoming greater.

No attempt has been made to calculate the results in terms of oxygen uptake per g. of tissue, since it was found consistently that under the conditions of these experiments, the oxygen uptake per g. of tissue is dependent on the amount of tissue taken. Using two different weights of the same tissue, the uptake per g. was always greater with the greater weight of tissue. Possibly some oxidative catalyst diffuses out of the cell into the buffer, and the velocity of oxygen uptake of the tissue depends on the concentration of this substance in the surrounding fluid. It is consequently difficult from the results in Tables I and II to calculate average figures for the oxygen uptake per g. of scorbutic and normal tissues. A further difficulty is that the time elapsing between the death of the animal and the first measurement varied in different experiments. On the whole, however, in comparing experiments in which equal or approximately equal weights of liver were used, it appears that the oxygen uptake of the scorbutic tissue is definitely lower than that of the normal tissue. The addition of ascorbic acid tends to raise the uptake of the scorbutic tissue towards that of normal tissue rather than to increase the uptake above the normal value.

A few experiments have been carried out on muscle, but these experiments have been less satisfactory, since I have been unable to prepare thin slices of muscle, and when chopped muscle is used a great part of the oxygen uptake occurs while the apparatus is being brought into equilibrium in the thermostat. The experiments indicate, however, that both with 0.1 mg. and 0.25 mg. of ascorbic acid, there is a slight increase in the oxidation of the muscle of normal animals and a relatively large increase in the oxidation of muscle taken from scorbutic animals, though too few experiments have been done to justify a quantitative comparison.

Recent experiments by Harris and Ray [1933] indicate that the guinea-pig requires about one mg. a day of vitamin C for normal maintenance. It seems therefore that in the experiments that I have described, in which the effects in oxidation have been produced *in vitro* by the vitamin in concentrations of 0.08 mg. per cc. or less, the vitamin is acting in concentrations which are not unphysiologically large.

That these findings have some relation to events in the intact animal is indicated by a recent paper by Söderström and Törnblom [1933], in which it is shown that feeding guinea-pigs on a scurvy-producing diet results in a marked decrease in the uptake of oxygen by the intact animals.

It is probably premature to attempt to speculate on the relation of these findings to scurvy as a disease. It is not difficult to imagine, however, that impeded oxidation in the scorbutic animal might lead to loss of tone in the capillary muscles, leading to increased permeability and the characteristic haemorrhages associated with scurvy.

Tissues other than liver and muscle have not so far been examined. Gavrilescu *et al.* [1932] have shown that the action of vitamin B₁ concentrates in restoring the lowered oxygen uptake of polyneuritic pigeon's brain is specifically concerned with the glucose-lactate enzyme systems. Experiments designed to determine what oxidising system or systems are linked up with the activity of vitamin C are now in progress.

SUMMARY.

The surviving respiration of tissues taken from normal and scorbutic guinea-pigs was measured manometrically in oxygen, both with and without addition of vitamin C (ascorbic acid). With scorbutic animals, using thin slices of liver suspended in phosphate buffer (p_H 7.4), the oxygen uptake is lower than that of liver from normal animals.

The addition of 0.25 mg. of ascorbic acid in 3 cc. brings about an increase in the oxygen uptake of scorbutic liver slices amounting to 5 to 57 % (average 25 %). With normal guinea-pigs, the oxygen uptake of the liver was unaffected or even slightly decreased by the addition of 0.25 mg. of ascorbic acid. Using chopped muscle, the oxygen uptake with normal guinea-pigs was slightly increased with 0.1 to 0.25 mg. ascorbic acid, while with muscle from guinea-pigs suffering from scurvy, a relatively large increase in oxygen uptake was obtained.

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