XCI. THE REVERSIBLE ENZYMIC OXIDATION OF VITAMIN C.

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SOME years ago during the progress of an inquiry on the production of an antiscorbutically active cider from Bramley's Seedling apples it was observed that the juice expressed from the fruit while retaining a very high proportion of its antiscorbutic activity did not reduce indophenol. At that time the writer held the view that the capacity for reducing this indicator was mainly due to a substance which, although closely associated with the vitamin in a protective way, was not the active principle itself. The probability of the production of a reversibly oxidised active form of the vitamin was not at that time seriously considered by him.

This problem was reinvestigated recently in view of the fact which has now been established that *l*-ascorbic acid possesses antiscorbutic activity *per se* and that when this compound is reversibly oxidised [Szent-Györgyi, 1928] it retains its activity [Tillmans *et al.*, 1932; Hirst and Zilva, 1933].

It was found again that juice expressed from Bramley's Seedling apples although unable to reduce indophenol was antiscorbutically active. Thus a daily dose of 3 ml. of the non-reducing apple juice afforded protection to guinea-pigs equivalent to 0.25–0.5 mg. of ascorbic acid or to rather less than 1 ml. of decitrated lemon juice (10 I.U.). This potency is not very much less than that obtained with intact tissue of these apples (usually about 15 I.U. per 3 g.). The apple juice, it was further found, reduced indophenol immediately after expression but rapidly lost this power of reduction. Aqueous extracts prepared with or without cyanide, unlike alcoholic, trichloroacetic acid and strongly acid extracts did not reduce indophenol. Furthermore, the non-reducing apple juice after treatment with hydrogen sulphide regained the property of reducing indophenol. This evidence is very suggestive of a case of enzymic reversible oxidation. Experiments will here be described which strongly support this hypothesis.

EXPERIMENTAL.

The oxidation of vitamin C in lemon juice.

15 ml. of freshly expressed apple juice (Bramley's Seedling), which did not reduce indophenol, were added (a) to 50 ml. of decitrated lemon juice p_H 7 (resulting p_H of mixture, 4.4); (b) to 50 ml. of decitrated lemon and the mixture adjusted to p_H 3 (reaction of apple juice). These mixtures were allowed to remain in the presence of air at room temperature for 6 hours. At the end of this time the reduction in (a) had fallen from 4.9 to 0.8 ml. *N*/1000 indophenol per ml.; in (b) from 4.9 to 2.1 ml. of the indicator. The corresponding control solutions of decitrated lemon juice which did not contain any apple juice lost very little of

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their indophenol reducing power. The oxidised solutions were then submitted to reduction with hydrogen sulphide as described by Johnson [1933]. The titre of (a) and of (b) rose in consequence to 6 ml. *N*/1000 indophenol. These figures were higher than the original (4.9 ml.) owing to the fact that the oxidised apple juice in the samples was also reduced in the process. An experiment performed with pure ascorbic acid in aqueous solution gave similar results.

The biological activity of the reversibly oxidised vitamin C in lemon juice.

Decitrated lemon juice treated daily as above and kept at p_H 4.4 was used in these tests. The indophenol reducing power left in these preparations after they had stood for 6 hours varied with the samples of the apple used. Only very seldom was the reducing power found to have disappeared entirely. Occasionally oxidation was not very advanced after this time. In such cases the preparation was allowed to remain for about 24 hours, after which no reducing power could usually be observed. It was, however, considered inadvisable to test 24-hour samples daily in order to avoid the possibility of considerable direct oxidation by atmospheric oxygen. Daily doses of 1.5 ml. of the oxidised preparations were tested, and decitrated lemon juice of an equivalent reducing capacity was administered to a control group of guinea-pigs. It will be seen from Fig. 1 that the

Fig. 1.

C = Chloroformed.

+ = Died.

S = Scurvy.

V.S.S. = Very slight scurvy.

N = Normal.

↓ = Onset of clinical symptoms of scurvy.

A. These animals received 1.5 ml. of reversibly oxidised decitrated lemon juice daily. Average reduction value of daily dose 1.4 ml. *N*/1000 indophenol.

B. These animals received as a control untreated decitrated lemon juice. Average reduction value of daily dose 1.8 ml. *N*/1000 indophenol.

reversibly oxidised dose of 1.5 ml. which showed an average daily reducing capacity of only 1.4 ml. *N*/1000 indophenol protected the animals to a very marked extent, whilst a dose of decitrated lemon juice of a similar reducing

capacity afforded hardly any protection. As the activity of apple juice present in the oxidised dose was negligible it may be assumed that the reversibly oxidised vitamin of the decitrated lemon juice was mainly responsible for the protection.

The influence of the absence of oxygen on the action of apple juice on vitamin C.

Experiments similar to the above were carried out at p_H 4.4 in an atmosphere of nitrogen. In a typical experiment the solutions were placed in ampoules which were then alternately exhausted and filled with nitrogen three times. These solutions as well as the controls, which were kept in conical flasks in the air, were stored side by side at room temperature for 24 hours. While the reducing power of the control solution fell from 5.5 ml. $N/1000$ indophenol to nil per ml. that of the anaerobic sample fell only to 4 ml. $N/1000$ indophenol. The small loss in reducing power in the latter was most probably due to manipulation, and reversible oxidation, if any, was therefore small.

The influence of boiled apple juice on vitamin C.

Experiments similar to the above were performed in which the apple juice used was previously boiled. The following is a typical experiment in which the reducing capacity of the sample containing the boiled apple juice fell at p_H 4.4 from 4.9 to 3.8 ml. $N/1000$ indophenol per ml. of solution in 6 hours, a fall which was very probably due to atmospheric oxidation. In the sample with the unboiled juice the original titre fell to 0.8 ml. $N/1000$ indophenol per 1 ml. of solution.

The influence of boiled apple juice with added peroxidase on vitamin C.

The apple juice used in the above experiments is capable of oxidising the usual peroxidase reagents in the presence of hydrogen peroxide. This oxidative activity is destroyed on boiling, and it was of interest to discover whether the addition of peroxidase to boiled apple juice would restore its capacity to oxidise vitamin C. The preceding experiment was therefore repeated with and without the addition of turnip-peroxidase to the boiled apple juice. It will be seen from the following representative experiment that this addition did not restore the oxidising activity of the boiled apple juice. Thus at p_H 4.4 the solution containing the boiled apple juice alone and the solution containing the boiled apple juice *plus* a few drops of turnip-peroxidase solution, as well as the control decitrated lemon juice containing no apple juice, fell in their reducing capacity from 5.1 to 4.5 ml. $N/1000$ indophenol per ml. of the solution.

The influence of cyanide on the action of apple juice on vitamin C.

As cyanide inhibits peroxidase activity, an endeavour was made to ascertain whether this reagent would also inhibit the oxidising activity of the apple juice. In these experiments carried out at p_H 4.4 the usual quantities of decitrated lemon juice and apple juice were used. To one sample sodium cyanide was added to make the concentration $M/500$. This concentration inhibited the peroxidase activity in the solution. The following is a typical example. In the sample containing cyanide the indophenol reducing capacity fell from 5.3 to 1.8 ml. $N/1000$ indophenol per ml. in 6 hours. In the control sample which did not contain any cyanide the reducing capacity fell to 1.1 ml. in the same time. This concentration of cyanide, therefore, had no serious inhibiting effect on the oxidation of the vitamin. Only when the concentration was raised ten- or twenty-fold was a marked inhibition observed.

DISCUSSION.

The experiments described supply fairly convincing evidence that the apple contains an enzyme which is capable of reversibly oxidising vitamin C without affecting its antiscorbutic potency to any very marked extent. The facts that peroxidase when added to boiled apple juice is incapable of oxidising the vitamin and that quantities of cyanide which inhibit this enzyme do not seriously interfere with the oxidation of the reduced form of the active principle, show that this oxidation is not brought about by peroxidase either alone or as one of the enzymes in a coupled system. The oxidising enzyme seems to resemble the one described by Szent-Györgyi [1931].

This worker found that unboiled, but not boiled, cabbage pulp rapidly lost its capacity for reducing Folin's phenol reagent and for taking up oxygen in a respirometer. Further, the addition of hexuronic acid (ascorbic acid) to cabbage juice greatly increased the uptake of oxygen. He concluded that cabbage contained an enzyme (hexoxidase) which could oxidise reversibly hexuronic acid. The oxidised product obtained by him was not tested for its antiscorbutic activity, as at that time the identity of ascorbic acid with vitamin C was not known. It is, however, quite conceivable that the oxidised product in cabbage juice is also active.

That the oxidising enzyme functions under physiologically controlled conditions in the tissue of the intact apple as well as in the disrupted fruit is more than probable. In fact, evidence which is very suggestive in this respect has been obtained during the last few years in an enquiry on the vitamin C of the apple by the writer in collaboration with Drs F. Kidd and C. West of the Low Temperature Research Station, Cambridge. The knowledge of the distribution of this enzyme in the plant and animal kingdoms would evidently greatly contribute to the solution of the problem of the function of vitamin C in plant and animal organisms.

SUMMARY.

The apple (Bramley's Seedling) contains a thermolabile enzyme which is capable of reversibly oxidising vitamin C without seriously impairing its antiscorbutic activity.

This enzyme does not function under anaerobic conditions and is only inhibited by very high concentrations of cyanide.

The peroxidase of the apple does not seem to be involved in this oxidation process.

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