CLXXX. FURTHER OBSERVATIONS ON THE SYSTEM ASCORBIC ACID-GLUTATHIONE- ASCORBIC ACID-OXIDASE

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In a paper by Hopkins & Morgan [1936] it was shown that in aqueous solutions containing Szent Györgyi's oxidase together with ascorbic acid and glutathione, the former is protected by the latter from oxidation by the enzyme, while the glutathione is itself oxidized vicariously at a rate corresponding to that at which the ascorbic acid is oxidized when alone with the enzyme in similar concentrations. It was also shown that under the influence of similar enzyme preparations the reduction of dehydroascorbic acid by GSH is much faster than the oxidation of ascorbic acid, and also much faster than the reduction when uncatalysed.

Kertesz has since stated [1938] that he was unable to reproduce these experimental results. He found that GSH added to solutions of ascorbic acid containing the oxidase had no effect on the oxidation of the ascorbic acid at pH 6. At pH 7.4, while the GSH inhibited to some extent the oxidation of ascorbic acid, both substances were oxidized simultaneously. He found further that the reduction of dehydroascorbic acid by GSH was not catalysed by the enzyme preparation used by him.

It became clearly desirable that the experiments should be repeated, and the present paper deals with such repetitions together with some extensions. They have involved the use of many different enzyme preparations, and they show that the results published by Hopkins and Morgan are invariably reproducible. The enzyme preparations used by these authors were all derived from cabbages or from cauliflower florets. Kertesz employed cauliflowers, but also used cucumber juice. With no preparation did he obtain results comparable with those of the earlier workers. In the following sections experiments with cauliflower florets will be first described; certain others obtained with cucumber juice will afterwards receive special reference.

Unless an explanation which we will later venture to put forward is justified, we seem to be faced with an inexplicable difference in experimental experience.

EXPERIMENTAL

The cauliflowers employed have been both English-grown and imported. The concentration (or activity) of the oxidase has varied widely in different cases. In general the fresher the source of the supply the greater was the activity of the juice, but it varied apparently with the degree of maturity of the florets. The juice was expressed with avoidance of contact with Fe or Cu, and glass-distilled water was always used for making up solutions. The juice in each case was centrifuged before use, but was not otherwise fractionated. Owing to the wide variations in enzyme concentration we found it desirable to make a preliminary estimation of the rate at which each preparation oxidized a known

amount of ascorbic acid before proceeding to other experiments. The GSH was prepared in the laboratory; crystalline fractions almost free from any tendency to autoxidation being always employed. The ascorbic acid was usually that supplied by the British Drug Houses Ltd., but we have also used a standardized natural product kindly supplied by Sir Henry Dale. Both reactants were more stable than were apparently those employed by Kertesz. The methods of estimation were exactly those used by Hopkins & Morgan and also by Kertesz. A minor difference in procedure was that while our solutions were shaken continuously on a machine, in Kertesz's experiments they were shaken every 2 min. by hand. Our supply of GSSG was made by enzymic oxidation arrested before irreversible products were formed.

Experiments with cauliflower juice

In the work of Hopkins and Morgan, the behaviour of the system—ascorbic acid-GSH-oxidase—was chiefly studied at pH 7.4. It seemed desirable to determine through what range of variation in pH the protection of ascorbic acid and the other results obtained by them (if confirmed) would remain constant. Experiments were therefore made at pH 4.5, 6.0, 6.75, 7.4 and 8.25 respectively. The results are shown in Figs. 1-5.

Fig. 1 shows the relations displayed during aeration at pH 7.4. The enzyme preparation used (from a home-grown cauliflower) was initially very active, 50 mg. ascorbic acid (or, in the system an equivalent amount of GSH) being oxidized in < 20 min. It was found that the oxidation of the ascorbic acid began just when a nitroprusside test first showed the disappearance of GSH. In order however that the relations could be more clearly shown (Fig. 1), the juice was diluted before use with twice its volume of glass-distilled water to reduce the velocity of change. The results reproduced exactly those of the earlier studies.

Fig. 1.

Fig. 2.

Fig. 1. Behaviour of system at pH 7.4. Upper line shows protection and subsequent oxidation of ascorbic acid. Lower line, course of oxidation of GSH in system. 1.75 mol. of former to 1 mol. of latter. McIlvaine's buffer (phosphate-citrate).

Fig. 2. System at pH 8.25. 2 mol. GSH to 1 mol. AA. Upper line, protection and subsequent oxidation of ascorbic acid. Middle line, oxidation of GSH in system. Lowest line, oxidation of ascorbic acid with enzyme in absence of GSH. McIlvaine's buffer.

The results at pH 8.25 are shown in Fig. 2. They were given by an undiluted preparation and are essentially the same as those obtained by Hopkins & Morgan, and by ourselves, at pH 7.4.

Fig. 3 shows the behaviour of the system at pH 6.75 and Fig. 4 that at pH 6. Both are again identical in essentials with the above.

Very different is the behaviour at pH 4.5 (Fig. 5). At this more acid reaction the system is dislocated; the protection of the ascorbic acid is imperfect and (as in Kertesz's experiments) both it and GSH are oxidized together.

Fig. 3.

Fig. 4.

Fig. 3. System at pH 6.75. Details as in Fig. 1. GSH/ascorbic = 2 mol./1 mol.

Fig. 4. System at pH 6. Details as in Fig. 3.

It is easy to show from pH-velocity curves that this is because somewhere near pH 5 there is a critical point at which the velocity of the reducing process falls below that of the oxidative one.

Fig. 5.

Fig. 6.

Fig. 5. System at pH 4.5. Two different enzyme preparations. Upper curve in each case, oxidation of GSH. Lower curves that of ascorbic acid. Ratio GSH/ascorbic = 2 mol./1 mol. McIlvaine's buffer.

Fig. 6. Lower line, enzymic oxidation of ascorbic acid; upper line, catalysed reduction of dehydro-ascorbic acid by GSH. For details see text. Sørensen's or McIlvaine's buffer.

Before the behaviour of the system at these low pH values can be understood, the behaviour of the mechanism which catalyses the reduction of ascorbic acid by GSH must receive reference. Its existence was clearly shown in the experiments described by Hopkins & Morgan, but Kertesz states that he was unable to repeat them. Pfankuch [1934] found that in potato juice the reduction of

dehydroascorbic acid by cysteine required enzymic catalysis for its occurrence as it did not occur in the previously heated juice. The influence of such a catalyst was first clearly shown in the experiments just mentioned.

Fig. 6 shows the relative velocities of the oxidation of ascorbic acid and its reduction by GSH as induced by a preparation of cauliflower juice. 88 mg. natural ascorbic acid in 100 ml. Sørensen's phosphate buffer at *pH* 6, together with 10 ml. enzyme preparation, were shaken in an open flask. The course of oxidation was followed by titrating successive 10 ml. samples. Oxidation was stopped at a point fixed by extending the strictly linear course to the base line when it could be assumed that reversible oxidation was complete. Samples of 10 ml. each were then transferred to a series of vacuum tubes in the bent-over hollow stoppers of which were 30.7 mg. GSH in 2 ml. buffer. After evacuation the GSH was mixed with the solutions and the tubes allowed to stand at room temperature (21°). The contents of the individual tubes were then titrated at successive time intervals. Oxidation and reduction thus progressed under the enzyme as originally added, but the volume of solution during reduction was greater than during oxidation in the proportion of 12 : 10. It should be noted that in this experiment only 2 mol. GSH were added for each mol. ascorbic acid; yet the reduction rate was linear until the process was almost complete, just as in the experiments which demonstrate that protection lasts till the GSH is practically all oxidized.

The uncatalysed reduction of ascorbic acid by GSH is a phenomenon long familiar in this laboratory. That it is much slower than the catalysed reaction was shown by an experiment—one of many with similar bearings—of which the results are given in Fig. 7. In this case the GSSG was made by oxidizing GSH with iodine. The I_2 was quantitatively removed by lead acetate and a minute excess of Pb by K_2SO_4 . Reduction was carried out in vacuum tubes; 4 tubes in each of 3 series. One series contained the enzyme solution, in a second this was replaced by an equal volume of water, in the third each tube contained 0.022 mg. Cu. Otherwise the content of every tube was the same, 3 mol. GSH being present for each mol. ascorbic acid. The *pH* was 6. The evacuated tubes stood at room temperature (22°) and their contents were titrated at successive intervals. In Fig. 7 the upper curve shows the rate of the enzyme-catalysed reduction, the lower curves that of reduction in the absence of enzyme. Of the latter two, which are nearly identical, the lower shows that Cu does not catalyse the reduction process. With enzyme the dehydroascorbic acid was completely reduced in 15 min. or less, without it only 40 % was reduced in 1 hr. It is the experience of this laboratory that similar results are always to be obtained with the two varieties of *Brassica* that have so far been employed. In his paper Kertesz [1938] remarks "The results obtained with the system shown in their Fig. 3 (i.e. complete protection of the ascorbic acid so long as GSH is present) were observed at *pH* 7.4. It is doubtful whether the experiment could have been repeated at *pH* 6, although Hopkins & Morgan make no statement to this effect." Fig. 6 of the present paper shows however that there is complete protection at that *pH* while the catalysis of reduction at *pH* 6 is fully confirmed.

It is important to note the general effect of *pH* on the relative activities of the oxidase and the reducing enzyme (or catalytic system).

Fig. 8 is based on determinations of the initial velocities of oxidation and reduction respectively at varying *pH* values. They were made with an active specimen of cauliflower juice. The flatter and unsymmetrical curve (*A*) relates to oxidation velocities, the symmetrical (*B*) to those of reduction. The points on the curves are from estimations of the amount of change in either direction

induced in 15 min. It is seen that through a range of high pH values reduction is a faster process than oxidation while through a range of lower values reduction is the slower. In the complete system protection of ascorbic acid during the presence of GSH would be expected throughout the former range and not within the latter. In a number of less complete studies we found that the transition point where the curves cross lay between pH 4.5 and 5. The exp. of Fig. 8 was

Fig. 7.

Fig. 8.

Fig. 7. Curves run from right to left. Upper, the catalysed reduction by GSH. Lower continuous line, the rate of reduction when uncatalysed. Lower broken line, rate of reduction in presence of copper. Sørensen's buffer.

Fig. 8. pH -velocity curves. Curve *A* gives the initial rates of oxidation measured at different pH values. Curve *B* applies similarly to rates of reduction in the presence of the enzyme of (enzymically prepared) dehydroascorbic acid. McIlvaine's buffer.

carried out for the construction of more complete curves for publication, and it happened that the transition point in this case was just above pH 5. It would indeed be satisfactory if all the aspects of behaviour displayed by the system could be studied with the same preparation and without delay. This we have found to be impossible. While it is true, as various authors have stated, that the oxidase itself is relatively stable, the reducing mechanism is much less so. Its activity may be lessened in preparations kept overnight, and falls off slowly when an expressed juice is shaken in the air in the absence of any added substrate.

From these considerations it will be understood that in observations made at a pH only a little above 5, the degree of protection may be uncertain. At pH 5.25 for instance we have obtained typical protection with a fresh juice, but none with the same juice after standing for 3 days, though even then there was sufficient of the reducing factors left for protection at pH 7.4.

Fig. 8 makes clear the general nature of the influence of pH variations on the relative rates of oxidation and reduction, and explains completely the disappearance of protection at pH 4.5 (Fig. 5).

Hopkins & Morgan [1936] reported a circumstance involving some difficulty in explanation. Though typical protection was obtained in the system when dialysed juice was employed, dialysis nevertheless diminished the power of preparations to promote reduction. Kertesz [1938] in commenting on this remarks, "The observation that dialysed cauliflower juice could produce complete protection of ascorbic acid in the presence of glutathione but could not catalyse the reduction of dehydroascorbic acid by glutathione makes the explanation given for the mechanism somewhat uncertain." This does not state the facts correctly. With dialysed juice the initial velocity of reduction was as

great as with undialysed, but the rate fell off before reduction was complete. This circumstance still lacks explanation, but the additional evidence given in this paper for the relative instability of the reducing factor (or factors) perhaps affords some help. It is possible that dialysis reduces the amount of some protective agent. As stated above, the reducing activity of a preparation falls off when it is shaken aerobically by itself. This destruction may proceed faster in the dialysed juice.

We venture to include here parenthetically the results of an experiment carried out by Hopkins & Morgan but not published, which shows how justly ascorbic acid might be looked upon as a coenzyme for the oxidation of GSH. Dialysed juice was employed so that all pre-existing ascorbic acid was removed and GSH therefore quite unaffected by the oxidase. In Fig. 9 is shown the effect of subsequently adding minute amounts of ascorbic acid on the rate of GSH oxidation.

Fig. 9.

Fig. 10.

Fig. 9. Exp. with dialysed juice. Broken line, GSH with enzyme alone (unaffected). Middle line, oxidation of same in presence of 0.1 mg. ascorbic acid. Lower line with 1.0 mg. Sørensen's buffer, pH 7.4.

Fig. 10. System at pH 7.0 with cucumber juice. Upper line, oxidation of ascorbic acid in system. Middle curve, oxidation of GSH in system. Left-hand line, oxidation of ascorbic acid when alone with enzyme. 2 mol. GSH to 1 mol. ascorbic acid. McIlvaine's buffer.

Experiments with cucumber juice

Until the appearance of Kertesz's paper we had had no experience of the use of preparations from the cucumber, which were used in the greater number of his experiments. It is well known that enzyme systems differ widely in plants of different families, and they may well differ in different tissues of the same plant. One thing is sure, however. Unlike some other fruits, e.g. the apple [Johnson & Zilva, 1937], the cucumber contains the ascorbic acid oxidase [Kertesz, 1938]. It also contains the reducing system, but in all the specimens we have employed the concentration of this relative to that of the oxidase is small when compared with their mutual relations in the leaves and florets of the *Brassica* plants. In experiments such as those on which Fig. 8 was based, we have found that the velocity of reduction never rises nearly so far above that of oxidation as is shown in this figure, and it remains above it during a much smaller range of pH values. The maximum velocity of reduction is at a somewhat less acid reaction (about pH 7) than that of oxidation. It further seems sure that for some reason the reducing system is less stable or less protected in

cucumber juice than in that of cauliflower or cabbage. This indeed seems to be the case with the oxidase also. When the two juices without any addition are thoroughly aerated side by side by shaking at pH 6, the resultant falling off of oxidase activity is much more marked in the cucumber juice than in the other. It may be however that a lower relative concentration of the reducing system in cucumber constitutes the main difference between them.

The results we have obtained with cucumbers do not differ fundamentally from those published by Kertesz, except that at pH values near to 7 we have found greater protection by GSH than he reported. At pH 6 it may be small or absent. Fig. 10 gives results typical of those we have obtained at pH 7.0. The departure of the GSH oxidation from the linear progress which was always found in the experiments with cauliflower juice may have been due here to a gradual destruction of the less stable reducing system.

DISCUSSION

In this paper it is shown that all the results obtained by Hopkins & Morgan in their study of the system under reference are reproducible, and we may add that when the experimental conditions are properly defined the reproduction has been in our experience invariable. In so far as he used the same materials as those used by them, the inability of Kertesz to obtain the same results is exceedingly difficult to explain.

The greater number of Kertesz's experiments were however carried out with preparations from cucumbers, and with this material our own results, though not identical with his, come closer to them. It is so well known that the enzymes in plants of different orders or families vary in nature and organization, and so probable that those in different tissues even of the same plant may differ, that it was not justifiable for this author to apply results obtained from material different from that used by Hopkins & Morgan in criticism of their findings. This he does, at least by implication.

It is true that he also used cauliflowers, but apparently in a few experiments only. There remains nevertheless the difficulty of reconciling his results with those of Hopkins & Morgan and our own. It is perhaps possible though very improbable that the explanation might be found in the different provenance of the plants employed; Kertesz's experiments being done in Stockholm. This becomes the less likely since in the Cambridge experiments plants imported from the Continent and others grown on different soils in England have been employed. A more likely explanation is to be found in the instability of the reducing mechanism, of which Kertesz was not aware. He has kindly informed us that the plants used had probably travelled a long distance before they were purchased, and that the expressed juices were not always used immediately after their preparation.

We are particularly concerned to emphasize the quite certain presence in the plants which, because of the nature of Szent Györgyi's pioneer studies, were used by ourselves, of an agent which actively catalyses the reduction of ascorbic acid by glutathione. Its distribution has yet to be explored, but even if this proves to be limited, it is yet an enzyme (or enzyme in association) of which the kinetics must possess a special interest. In any study of these its relative instability must be remembered. An endeavour is being made in this laboratory to separate it from the associated oxidase.

SUMMARY

The experiments of Hopkins & Morgan [1936] on the system ascorbic acid-glutathione-ascorbic acid-oxidase, which Kertesz [1938] was apparently unable to confirm, have been repeated and extended, and the original results completely confirmed. In particular the effect of variations in *pH* on the enzymic activities of oxidase preparations has been determined.

The expressed juice from cucumbers differs in certain noteworthy respects from that obtained from cabbages and cauliflowers. It contains the oxidase, as Kertesz has shown, and it also contains a mechanism capable of catalysing the reduction of ascorbic acid by glutathione. The latter however is present in relatively low concentration.

Possible reasons for Kertesz's inability to repeat the earlier experiments are discussed.

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