LXXXII. OBSERVATIONS ON THE SYSTEM ASCORBIC ACID-GLUTATHIONE- ASCORBIC ACID OXIDASE

By Z. I. KERTESZ

*From the Biochemical Institute, University of Stockholm, and the
New York State Agricultural Experiment Station, Geneva, N.Y.*

(Received 15 January 1938)

ASCORBIC acid and glutathione are both highly reducing substances found in living tissues. In the absence of metallic or other catalysts they are not oxidized by atmospheric oxygen. Szent-Györgyi [1928; 1931] described an enzyme which oxidized ascorbic acid but had no effect on glutathione. But if ascorbic acid, glutathione and ascorbic acid oxidase were present simultaneously, oxidation of the glutathione occurred because the enzymically produced dehydroascorbic acid was reduced by the glutathione which was meanwhile itself oxidized.

This interesting interrelation of the three substances was the subject of a later investigation by Hopkins & Morgan [1936]. According to these authors, in the system containing the three components at pH 7.4 the ascorbic acid was completely protected from oxidation by the presence of glutathione. Meanwhile the glutathione was oxidized at a rate corresponding to that at which the oxidation of ascorbic acid alone would have proceeded under the influence of the same quantity of enzyme. When the oxidation of the glutathione was complete, and only then, did the oxidation of the ascorbic acid begin. This was only possible if the dehydroascorbic acid were reduced by the glutathione and if this reduction were more rapid than the enzymic oxidation of the ascorbic acid.

In the course of their work Hopkins & Morgan also described the presence of an enzyme in cauliflower juice which catalysed the reduction of dehydroascorbic acid by glutathione. The object of the present study was to investigate this latter reaction. As shown by the results presented below no such enzyme could be found in the cauliflower and cucumber juices used, nor could Hopkins & Morgan's observation regarding the complete protection of ascorbic acid from oxidation by glutathione be confirmed.

EXPERIMENTAL

Materials and methods

During this investigation the utmost care was taken to avoid contamination by metals of the materials entering the reaction mixtures. The distilled water was twice more redistilled from Jena glass before it was used. Chemicals of the highest available purity were used. As a result the autoxidation of ascorbic acid and of glutathione was negligible at pH 6.0. At pH 7.4 the autoxidation of the ascorbic acid did not exceed 10 % in 2 hr. At this latter pH about 15–20 % of the glutathione was autoxidized in 1 hr. and 35–40 % in 2 hr. This blank was also observed when the glutathione was held at pH 7.4 in the buffered solution without ascorbic acid or enzyme. Crystalline natural ascorbic acid and glutathione from F. Hoffmann-LaRoche and Co. were used.

The juices were pressed between hardwood plates in a hand screw-press and did not come into contact with metal parts at all. The cauliflower juice had a similar oxidase activity to that used by Hopkins & Morgan, while the cucumber juice was about twice as active on the volume basis. The different centrifuged samples of cauliflower juice contained about 4% total solids, the cucumber juice about 3%. The activity of the juices did not change materially when they were held in the ice-chest for a week or 10 days.

The methods given by Hopkins & Morgan were followed in all details unless noted otherwise. The percentages of total ascorbic acid and glutathione oxidized are shown in the figures rather than the actual titration figures. The values obtained in the control experiments were not subtracted from those presented in the figures because there is some uncertainty whether autoxidation (most likely from traces of metals) would occur to the same extent in the complete reaction mixture. All the experiments were carried out at 30°. Instead of using a shaking machine, the 50 ml. Erlenmeyer flasks containing the reaction mixtures were shaken by hand every 2 min. Preliminary experiments showed that the oxidation of ascorbic acid had the characteristic linear course. The differential titration with Tillmann's reagent and *N*/100 iodine was used for the determination of unchanged ascorbic acid and glutathione.

RESULTS

(1) *The ascorbic acid-glutathione-ascorbic acid oxidase system at pH 6.0*

A study of the system at this pH was not reported by Hopkins & Morgan. It was thought important to determine the changes which take place at the reaction most favourable for the enzyme. In the 10 ml. reaction mixture containing 0.5 ml. cucumber juice and 8 mg. ascorbic acid oxidation was complete in 27 min. When glutathione was added to the mixture at the rate of 2 mol. glutathione to 1 mol. ascorbic acid the velocity of the oxidation was hardly influenced at all and even after 2 hr. only 8% of the glutathione was found oxidized. These results are shown in Fig. 1.

Fig. 1. Enzymic oxidation of ascorbic acid (AA) by cucumber juice at pH 6.0.
(a) Without glutathione (GSH), (b) with GSH.

(2) *The system at pH 7.4*

In the absence of glutathione the oxidation of the ascorbic acid was complete in 36 min. Addition of glutathione retarded this oxidation and the glutathione was also oxidized. When the glutathione was added in a larger concentration (4 mol. to 1 mol. ascorbic acid) the protecting effect was much more pronounced. The results obtained are shown in Fig. 2. When the experiments were repeated with cauliflower juice the glutathione (2:1) gave somewhat greater protection.

In these experiments, the results of which are presented in Fig. 3, the concentration of the enzyme was also varied. In all experiments at pH 7.4 both the ascorbic acid and the glutathione were oxidized from the beginning of the reaction. Experiments with dialysed juices gave similar results.

Fig. 2. Enzymic oxidation of ascorbic acid (AA) by cucumber juice at pH 7.4. (a) AA without glutathione (GSH), (b) AA with GSH (2 mol. GSH: 1 mol. AA), (c) AA with GSH (4 mol. GSH: 1 mol. AA).

Fig. 3. Enzymic oxidation of ascorbic acid (AA) by cauliflower juice at pH 7.4. (a) AA with 0.5, 1 and 2 ml. enzyme, (b) 2 ml. enzyme and glutathione (GSH) (2 mol. GSH: 1 mol. AA), (c) as (b) but with 1 ml. enzyme, (d) as (b) but with 0.5 ml. enzyme.

(3) Attempts to catalyse the reduction of dehydroascorbic acid by glutathione with enzymes

The cause of the failure to repeat the experiments of Hopkins & Morgan may have been the absence from the reaction mixtures of a factor catalysing the reduction of the dehydroascorbic acid by the glutathione. Indeed, experiments with cucumber and cauliflower juices did not indicate the presence of such an enzyme in the material used. It was thought probable that the concentration of the glutathione was not sufficient to produce a rapid reduction. To obtain information on this point ascorbic acid was oxidized by cucumber juice in a manner similar to that described by Hopkins & Morgan in their experiment the results of which are shown in their Fig. 7. Afterwards 1 ml. samples of this reaction mixture were measured into Thunberg tubes containing 0.5 ml. buffer solution at pH 6.0. Then in small inner tubes different quantities of glutathione dissolved in 2 ml. water were placed, the Thunberg tubes were evacuated, their contents were mixed and the tubes were kept at 30° for different lengths of time. Afterwards the tubes were opened in such a way that they sucked in 2 ml. N HCl to acidify the mixture before air gained access to it. When they were fully opened their contents were diluted with a little water and titrated with Tillmanns's reagent. The results presented in Fig. 4 indicate that the increasing quantities of glutathione did not influence greatly the rate of reduction. It is also apparent that the rate of reduction was slower than the rate of oxidation.

After proving that the failure to obtain faster reduction was not caused by the small quantities of glutathione used, another possibility was considered. The quantity of enzyme solution in the reaction mixtures was only 0.025 ml. in the above experiment and 0.05 ml. when the experiment was repeated with cauliflower juice. In a further set of determinations the 1 ml. samples containing the

Fig. 4. Regeneration of oxidized ascorbic acid (AA) in presence of glutathione (GSH). (a) The course of oxidation at pH 6.0, (b) the reduction of dehydroascorbic acid (1 mol.) by 10 (curve 1), 20 (curve 2) and 40 (curve 3) mol. GSH at pH 6.0. The point marked X at 80 min., indicating 70% recovery of AA, was obtained by the method of Borsook *et al.* [1937], using H_2S .

Fig. 5. Effect of additional enzyme on the regeneration of dehydroascorbic acid with glutathione (GSH). (a) The course of enzymic oxidation at pH 6.0, (b) the reduction by GSH (10 mol. GSH: 1 mol. AA) at pH 6.0 (curve 1), plus 0.1 ml. cauliflower juice (curve 2) and plus 0.1 ml. cucumber juice (curve 3).

oxidized ascorbic acid were mixed with 1 ml. glutathione solution and buffer, but in addition 0.1 ml. cucumber or cauliflower juice was added to them. These reactions were carried out in Thunberg tubes in the manner previously described. A glance at the curves in Fig. 5 reveals that the added enzyme did not hasten the regeneration by glutathione. On the contrary, the added juice inhibited the reduction slightly.

Experiments also established the fact that dehydroascorbic acid made by oxidation of ascorbic acid with Tillmanns's reagent was reduced by glutathione at a similar rate. Addition of cucumber or cauliflower juice did not influence the velocity of this reaction. The rate of reduction of dehydroascorbic acid by glutathione observed in the above experiments was similar to that described by Borsook *et al.* [1937] for corresponding concentrations at pH 6.0.

DISCUSSION

If glutathione completely protects ascorbic acid from enzymic oxidation and meanwhile glutathione is itself oxidized, the explanation suggests itself that the reduction of dehydroascorbic acid by glutathione is faster than the enzymic

oxidation of ascorbic acid. Hopkins & Morgan proposed this explanation and also presented some experimental data to prove its validity. While no better explanation for the facts observed can be offered by the writer, it should be pointed out that the evidence presented by Hopkins & Morgan was only indirect and partly contradictory. The results obtained with the system ascorbic acid-glutathione-ascorbic acid oxidase, shown by their Fig. 3, were observed at pH 7.4. It is doubtful whether the experiment could have been satisfactorily repeated at pH 6.0, although Hopkins & Morgan make no statement to this effect. In the experiment presented in Fig. 1 of this paper no protective action of glutathione could be observed. In their experiments the mol. ratio of glutathione to ascorbic acid was 2 : 1 and their results could be obtained with dialysed juice. On the other hand, their experiments to prove that the reduction of the dehydroascorbic acid by glutathione was faster than the oxidation of the ascorbic acid were made at pH 6.0 and could not be satisfactorily repeated at pH 7.4. Furthermore, the mol. ratios of glutathione to ascorbic acid were 4 : 1 and 8 : 1 and these experiments could not be repeated with dialysed enzyme solutions.

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 of the reaction somewhat uncertain. The additional observation presented in this paper that the protecting effect of glutathione is (in some cases at least) partial only and not complete makes it appear that some other factor than the enzymic catalysis of the reduction may be involved in the reaction.

It should be stated that the contradictory character of some of the results of Hopkins & Morgan was mentioned in their paper. The purpose of the present discussion is not to point these out but rather to contribute to our knowledge of this interesting but apparently rather complicated interrelation of the three substances. It is the author's desire, furthermore, to call attention to the fact that the observations of Hopkins & Morgan could not all be duplicated under conditions in which the only apparent difference was in the vegetable juices used as sources of ascorbic acid oxidase.

Further work is needed to explain the cause of this discrepancy as well as some of the points not entirely clarified by the work of Hopkins & Morgan.

SUMMARY

Glutathione added to solutions of ascorbic acid containing ascorbic acid oxidase (cucumber and cauliflower juice) had no effect on the oxidation of the ascorbic acid at pH 6.0. At pH 7.4 the glutathione inhibited the oxidation of ascorbic acid and both ascorbic acid and glutathione were oxidized simultaneously.

The reduction of dehydroascorbic acid by glutathione was not catalysed by the cucumber and cauliflower juices used in this work.

REFERENCES

- Borsook, Davenport, Jeffreys & Warner (1937). *J. biol. Chem.* **117**, 237.
Hopkins & Morgan (1936). *Biochem. J.* **30**, 1446.
Szent-Györgyi (1928). *Biochem. J.* **22**, 1387.
— (1931). *J. biol. Chem.* **90**, 385.