23. THE SYSTEM DEHYDROASCORBIC ACID-GLUTATHIONE

By ERIC MITCHELL CROOK

From The Biochemical Laboratory, Cambridge

(Received 23 January 1941)

THE ability of tissue extracts from plants and animals, either to inhibit the oxidation of ascorbic acid (hereafter 'AA'), or to reduce added dehydroascorbic acid ('DHA'), is well known. In his earliest experiments on AA (hexuronic acid), Szent-Györgyi [1928] showed that the peroxidase system of turnip juice would oxidize the natural AA in the juice when H_2O_2 was added, but that ascorbic acid was regenerated on standing. He also found that minced rat kidneys quickly reduced added DHA made by oxidizing AA with I_2 , and that this reduction was performed in buffer alone by SH glutathione ('GSH'). Similar results have been obtained by Strachitskii & Rubin [1936], who found that DHA injected into living cabbage leaves was reduced and greatly increased the respiration, and by Goldstein & Volkensohn [1938], who showed that in tumour, placenta, spleen and embryo tissue, the initial oxidation induced by Cu or Fe is followed by reduction back to AA. Also, Golyanitskii & Belonosov [1936] demonstrated that DHA from the oxidation of AA by heat and air is reduced by inclusion in an alcoholic fermentation.

Following Szent-Györgyi's suggestion of the rôle of GSH, Mawson [1935] explained the protective action of tissue extracts in preventing the destruction of AA partly, at least, by their content of GSH, whilst de Caro & Giani [1934] and Barron *et al.* [1936] showed that GSH was capable of completely protecting AA from oxidation by tissues or by Cu.

Bersin *et al.* [1935], however, were unable to reduce DHA with GSH. Their results are unexplainable and do not tally with those of any other workers. In fact, some of the characteristics of the reaction have been investigated by several workers, notably Borsook *et al.* [1937].

Pfankuch [1934] was the first to describe catalytic reduction of DHA by SH compounds. He showed the presence, in potato juice, of a heat-labile substance, precipitable by protein reagents, which catalysed the formation of AA from DHA and cysteine. Similar findings were reported by Hopkins & Morgan [1936]. GSH was found to prevent the oxidation of AA by the ascorbic oxidase of cauliflower and cabbage juice, but was itself vicariously oxidized in the process. They proved that this was due to a factor in the juice which catalysed the reaction between DHA and GSH, the rate of the uncatalysed reaction being not nearly high enough to account for the protection. Copper was not able to catalyse the reaction, so that its presence in the juice would not account for the effect. Szent-Györgyi [1931] had noticed the vicarious oxidation of GSH in the presence of AA and cabbage juice, but had not recognized its enzymic character. Martin [1940] has recorded a similar vicarious oxidation of 'virus protein' in the presence of AA, which he attributes to the SH groups of the protein and a reaction of the Hopkins-Morgan type.

Reducing catalysts were also demonstrated by Lohman & Sanborn [1937] in the juice of peas and beans, and by Rubin *et al.* [1937] in *Hydrangea*, *Cyclamen*,

Zea etc. The former workers have also confirmed Hopkins & Morgan's report that Cu is not responsible.

Fujita & Numata [1938], using pumpkin juice, and Kertesz [1938], using the juice of cauliflowers and cucumbers, were unable to repeat the observations of Hopkins & Morgan, but these have since been confirmed and extended by Crook & Hopkins [1938], who showed the pronounced effect of pH and the difference in behaviour between cauliflower and cucumber. The latter contained very little, if any, of the reducing catalyst.

This paper is a preliminary report of the separation of the reducing factor in cauliflower juice from the associated oxidase and the 'resynthesis' of the protection system of Hopkins & Morgan. The work is not nearly complete, but it was felt that what has been done should be put on record as it is not being continued for the time being.

EXPERIMENTAL

Enzyme. The source of enzyme has been the florets of cauliflower and broccoli, both English and foreign. These were minced and pressed and the juice centrifuged, and sometimes fractionated before use. Care was also taken to prevent access of Cu and glass-distilled water used throughout.

Dehydroascorbic Acid. Great difficulty was experienced in evolving a satisfactory and rapid method for making DHA. Attempts to prepare it in the solid state by the method of Moll & Wieters [1936] were not satisfactory. The product was never sufficiently pure and the impurities inhibited the reaction. Unfortunately, there has been no opportunity to try out another method, the details of which were very kindly supplied by Prof. Hirst of Bristol University.

Oxidation of AA by the oxidase plus atmospheric O_2 was used in many cases, as were Cu plus atmospheric O_2 , Cu plus H_2O_2 , and I_2 . The last was also unsatisfactory as the I^- ion inhibits the reducing system very strongly. The most satisfactory method finally evolved was to use a saturated aqueous solution of pure benzoquinone (recryst. from light petroleum B.P. $< 40^\circ$) in amount just less than the equivalent of the AA so that no quinone remained in excess. Quinone was shown to be a strong inhibitor of the reducing reaction whereas the quinol produced had no such effect.

Reaction velocity. The reactions were followed by two sets of methods. In the earlier experiments, AA was estimated by titration; later by a photoelectric procedure. To follow the course of the oxidation, 10 ml. of solution containing 10 mg. AA, 2 ml. buffer (*M*/5 Sørensen or McIlvaine, pH 6) and 1 ml. enzyme solution were shaken on a machine to ensure thorough aeration. Samples were removed at stated intervals, the enzyme destroyed with trichloroacetic acid and AA estimated by titration with *N*/100 I_2 . All measurements have been done at pH 6 as this value is near the peak of both oxidizing and reducing systems. Reduction of DHA was followed anaerobically as described by Hopkins & Morgan using 2.5 moles of GSH per mole AA (i.e. slight excess of GSH) and estimating AA by the micro-Tillman method [Birch *et al.* 1933; Hopkins & Slater 1935], because of the presence of GSH, which also reacts with I_2 . Alternatively, measurements were made aerobically. If DHA had been prepared by shaking with enzyme, no further addition of juice was made. *M*/100 KCN was now used to inhibit the oxidase completely, and, as shown below, this has no effect on the reducing system. Later, all measurements of AA were made by the direct photoelectric method of Evelyn *et al.* [1938], modified for use with larger quantities of ascorbic acid. The AA solution was diluted to contain between 2 and 10 mg. AA per 100 ml. and a strong dye solution—one giving an initial reading of

about 18 on the galvanometer scale—was used. For these stronger dye solutions it was necessary to construct a series of calibration curves covering different initial readings, as the straight line relationship breaks down. They are, however, very rapidly and easily made. A Wratten No. 63 filter was found best.

When employing the photoelectric method, the use of trichloroacetic acid for stopping the enzyme action was discontinued, as it was found to interfere with the readings by altering the dye. Instead, the enzyme was destroyed by first making the solution 30 % with respect to acetic acid and later diluting to give the 5 % recommended by Evelyn *et al.*

Again, when DHA was made by oxidizing AA with quinone, the quinol formed rapidly reduced the dye, making titration methods useless. However, the rate of reduction is slow in comparison with that caused by AA. Evelyn *et al.* have shown that the latter is complete within 5 sec. The reduction of dye by AA alone is thus easily found by extrapolation of the galvanometer readings.

Nature of the dehydroascorbic acid

It was at first suspected that the DHA formed by enzymic oxidation of AA differed from that obtained by simple chemical oxidation. GSH, anaerobically and in the presence of the juice, always reduced the latter form at only 60–70 % of the speed at which it reacted with the former. When DHA is prepared by oxidation with a reagent, or by using Cu as catalyst, either the reduced reagent or Cu remains in the solution as an 'impurity' which could conceivably act as an inhibitor. Table 1 shows the average velocity of reduction, by GSH in the presence of the juice, of DHA made by various methods, together with the velocities resulting from the addition of the corresponding 'impurity' to enzymically oxidized AA. Except in the case of large amounts of Cu (which cause precipitation in the juice), these are all higher than the rates with 'chemical DHA'. Thus the greater part of the inhibition is not due to the presence of such substances.

Table 1. *Velocity of reduction of DHA by GSH*

DHA prepared by oxidation with	Reaction rate	Substance added to 'enzymic' DHA	Reaction rate
Enzyme	100	—	—
I ₂ —I ⁻ ion still present	33	0.04 N I ⁻	80
I ₂ —I ⁻ precipitated by Pb	72	0.02 N I ⁻	89
Cu (20 mg./100 ml.)—H ₂ O ₂	40	—	—
Cu (20 mg./100 ml.)—H ₂ O ₂ , Cu removed by dithiocarbamate	61	Cu: 0.2 mg./100 ml.	100
Cu (1 mg./100 ml.)—O ₂	66	1.0 mg./100 ml.	94
		2.0 mg./100 ml.	80
		20.0 mg./100 ml.	37
Quinone	66	Quinol: 10 mg./100 ml.	100

In an endeavour to obtain some evidence on the matter, an attempt was made to determine the specific rotation of DHA from each source. This proved very difficult and the results must be regarded with some suspicion, both because the rotation of DHA is very sensitive to changes in pH and concentration and nature of ions, and because periods of more than 20–30 min. enzymic oxidation cause appreciable opening of the lactone ring at the pH used. The short time means that unoxidized AA is present and must be allowed for in calculating the rotation: the juice, too, has a small blank.

The results are set out in Table 2. In Exp. 1, the AA control, the rotation was determined in the presence of NaOH equivalent to the AA, *M*/10 phosphate

Table 2. $[\alpha]_D^{20}$ of dehydroascorbic acid

Exp. no.	Oxidizing agent	No. of repeats	Total specific* rotation	AA not oxidized (%)	Rotation of DHA*
1	Nil	3	23°	100	—
2	I ₂	3	54°	0	54° ± 1°
3	I ₂	4	39°	0	39° ± 1°
4	I ₂	5	32°	0	32° ± 2°
5	Cu-O ₂	5	26°	70	33° ± 2°
6	Oxidase	5	25.5°	70	31° ± 2°

* Calculated as AA.

buffer at pH 6, and 4 % trichloroacetic acid, to represent the conditions which obtain in the Cu- and enzyme-oxidized solutions after addition of trichloroacetic acid. The figure found; 23° for the D line, compares favourably with that given by Herbert *et al.* [1933] for the Hg yellow line 578 mμ, with pure AA in weakly acid solution. Exp. 2 is included for comparison with the following values, and is pure AA oxidized with I₂ with no additions, i.e. the solution then contains only DHA and HI. The figure again is in good agreement with that of Herbert *et al.* In Exps. 4–6, AA was neutralized with NaOH and the oxidation carried out in phosphate buffer at pH 6, during 20 min.; HI, if present, was neutralized by adding the theoretical amount of NaOH; after acidification with 40 % trichloroacetic acid to make the final concentration 4 % the protein coagulum was centrifuged out if present, and the rotation determined. In the experiments with oxidase, a blank was run with the juice and all conditions the same but no AA present. The values shown in the table are corrected for this blank, which amounted to c. 20 % of the total rotation. Exp. 3 was carried out as for Exps. 4–6, but the AA was not previously neutralized. It is included to show the effect of the extra amount of Na salts. After oxidation, an aliquot was taken on which unoxidized AA was determined by titration with I₂. The figures for the rotation of DHA in the presence of AA were calculated assuming that no opening of the lactone ring had occurred during the time occupied in oxidation. This is approximately the case, since Borsook *et al.* [1937] have shown that at pH 6 and 37°, only 15 % of DHA is hydrolysed in 1 hr. Here the temperature was c. 18°, so that the inaccuracy thus introduced would be within the experimental error. For convenience, DHA is calculated as the equivalent amount of AA.

As will be seen, the rotation of the enzymically prepared form does not differ significantly from that of two chemically prepared modifications. We must conclude, therefore, that there is probably no great chemical difference between them.

The possibility of there being some type of loose complex necessary for the activation of DHA, and slowly formed in the juice, was considered, but allowing chemically prepared DHA to stand anaerobically with enzyme for some time before adding GSH was found further to decrease and not to increase the velocity.

The explanation appears to be that an inhibitor is present, and that this is precipitated when AA and the juice are shaken in air to prepare DHA (much protein is precipitated under these conditions). This, of course, does not occur when chemically prepared DHA is used. Weight is added to this conclusion by the finding that heating the juice to 38° for 20–30 min. precipitates a considerable amount of protein and at the same time increases its ability to catalyse the reaction by as much as 20 %.

Separation of the reducing catalyst

It was found to be comparatively easy to obtain the reducing factor virtually free from the associated oxidase, and thus to show that they are distinct entities. Heating to 38–39° for 20–30 min. does not affect the oxidase and actually improves the reducing effect, as has been mentioned. This treatment removes much protein by denaturation and the muddy solution now becomes a clear yellow. On half saturating with ammonium sulphate, approximately 80 % of the oxidase is precipitated and only 30 % of the reducing factor. By raising the concentration of ammonium sulphate to 80 % saturation, the remainder of the 'oxidase' activity is thrown down, together with most of the reducing factor; the total recoveries of both oxidase and reductase being of the order of 90 %. Rapid dialysis of the 50–80 % saturated fraction causes loss of most of the remaining 'oxidase' activity (i.e. it is probably due to Cu^{++} ion), without appreciably lowering the activity of the reductase. Prolonged dialysis, however, affects the latter very considerably, the loss of activity being restored neither by heated juice nor by diffusate from raw juice. This indicates the inactivation of the reducing catalyst rather than the loss of a coenzyme-like compound. Repetition of the precipitation between the 50–80 % saturation limits removes more impurities and again gives a 90 % yield with no oxidase activity.

Properties of the reducing catalyst

The reducing system, either purified or in the plant, is rather labile. When plants are stored in the refrigerator for more than 2 or 3 days, they are generally found to contain little reducing activity. The purified preparation keeps its activity rather better, being reduced to about half in the course of a week. It is more thermolabile than ascorbic oxidase; exposure for half an hour to a temperature of 45° reduces its activity to half, whilst 55° destroys it entirely. On the other hand, 60° has almost no effect on the oxidase.

Extremes of pH reduce the activity. Even in buffer at pH 7.5 the activity is reduced to one third in 5 hr., and acids precipitate it completely [cf. Pfankuch, 1934].

Table 3. *Effect of inhibitors*

Final conc. of inhibitor	Reaction velocity	
	Reductase	Oxidase
Buffer alone at pH 6	100	100
$(\text{NH}_4)_2\text{SO}_4$: $M/4$	93	—
KI: $M/25$	78	100
$M/50$	89	100
Cu^{++} : $3 \times 10^{-5} M$	100	150
$1.5 \times 10^{-4} M$	94	—
$3 \times 10^{-4} M$	80	—
$3 \times 10^{-3} M^*$	37	—
Benzoquinone: $M/1000^\dagger$	61	—
Quinol: $M/1000$	100	—
$M/200$	100	—
Dithiocarbamate: $M/1000$	100	80
KCN: $M/1000$	100	0
$M/100$	100	—
H_2S : $M/1000$	100	0
$M/10$	110	—
NaF: $M/1000$	86	—
$M/100$	83	100
Na iodoacetate: $M/1000$	83	100
$M/100$	30	100

* Some precipitation of protein.

† Added before GSH.

In neither case is the effect reversible. The pH -optimum of 6.7–6.8 given by Crook & Hopkins is confirmed; see Fig. 1. The activity becomes very low below pH 4 and almost impossible to measure above pH 8 because of the rapid hydrolysis of DHA.

The effects of various inhibitors are set out in Table 3, the results for ascorbic oxidase being included for comparison. As will be seen, the reducing catalyst is fairly sensitive to neutral salts; e.g. even moderate concentrations of ammonium sulphate inhibit somewhat, whereas the oxidase is not at all affected. Similarly, quite small concentrations of Cu^{++} ion inhibit a little, although the effect of the strongest concentration shown can be explained, to some extent at least, by the fact that this amount of Cu is sufficient to fix about 40 % of the GSH present, if co-ordinated GSH is assumed to be no longer available for reduction. Such typical inhibitors of the oxidase as KCN and H_2S , which act in $M/1000$ concentration, do not affect the reducing system even at ten times that concentration. In fact the latter speeds up the reaction, apparently being able to act in somewhat the same manner as GSH.

Fig. 1.

Fig. 2.

Fig. 1. pH -velocity curve for reductase. $M/5$ phosphate-citrate buffer, 1 mg. per ml. dehydroascorbic acid, 4.5 mg. per ml. glutathione (2.5 moles per mole of dehydroascorbic acid), 0.6 mg. per ml. quinol (quinone was used to make dehydroascorbic acid), purified enzyme preparation.

Kinetics

The initial velocity of reaction is proportional to the concentration of catalyst preparation over a wide range, the rate falling off at higher concentrations (Fig. 2—unpurified enzyme). It has not been possible to obtain adequate curves of velocity as a function of concentration of DHA and GSH owing to a shortage of the latter material, but assuming DHA to be the substrate rather than GSH (an assumption only valid for the purpose of this preliminary calculation), the Michaelis constant has been found in a few preliminary experiments to be of the order of $2 \times 10^{-3} M$ for the purified product. This is to be compared with a Michaelis constant for the oxidase of the order of $2 \times 10^{-6} M$.

The straight line kinetics described by Hopkins & Morgan for the reducing mechanism are less evident when purer preparations are used and when the relative concentration of GSH is reduced from 4 moles GSH per mole DHA as

used by Hopkins & Morgan [1936] and Crook & Hopkins [1938] to the 2.5 moles per mole here employed. The course of the reaction is initially linear, but when the concentration of GSH is low the curves begin to tail off fairly early (Fig. 3, curve *A*). With 4 moles GSH, this tailing off is hardly perceptible at the concentrations of other reactants here used (Fig. 3, curve *B*). However, when the uncatalysed component of the reaction is allowed for, even 4 moles of GSH do not give a high enough concentration to prevent tailing off. The reaction is obviously extremely complicated, and as its measurement is made more difficult by the simultaneous occurrence of the uncatalysed reaction as well as the hydrolysis of DHA to 2-3-diketogulonic acid, the reasons for this behaviour have not yet been elucidated. Fortunately, the rate of the last-mentioned reaction is sufficiently slow at the pH used not to interfere unduly with the significance of the curves given.

Fig. 3, curve *C*, shows a typical curve for the course of the catalysed reaction, this being obtained from curve *A* by subtracting the values of curve *D*, which shows the course of the uncatalysed reaction under the same conditions.

Fig. 3.

Fig. 4.

The association of this enzyme with ascorbic oxidase in the protective mechanism for ascorbic acid discovered by Hopkins & Morgan was illustrated in two ways. Firstly, by roughly separating the system into two parts by ammonium sulphate fractionation of the juice, testing each separately and then the recombined pair; secondly, by constructing a wholly 'artificial' protection system from oxidase and reducing catalyst.

In the first case, prepared juice was taken and the course of oxidation of AA and of reduction of the DHA thus formed was followed as by Hopkins & Morgan (Fig. 4, curves *A*). A portion of this juice was now separated into 0-50 and 50-80 % saturated fractions with ammonium sulphate. As noted above, the first contains the bulk of the oxidase and only about 20 % of the reducing factor. The course of the curves (*B*) thus obtained show a similar velocity for the oxidase, but a slow reducing reaction. The curve for the uncatalysed reaction (*E*) is included to show that, although the observed reduction of DHA is moderately fast, about half of it is accounted for by the simple chemical reaction. When the

50–80 % fraction is tested, its oxidase content is so low (Fig. 4, curve *C*) that the time required would be so long that much of the DHA would be hydrolysed. Hence this fraction was tested for the reductase in the following way. Oxidation

Fig. 5.

Fig 6.

Fig. 5. Upper curve: ascorbic acid in the presence of glutathione. Middle curve: glutathione. Lower curve: course of the oxidation of ascorbic acid alone.

Fig. 6. Upper curve: ascorbic acid in the presence of glutathione. Middle curve: glutathione. Lower curve: course of the oxidation of ascorbic acid alone.

Fig. 7.

of AA was carried out by the oxidase fraction (curve *B*). Now, if the system is reconstitutable, addition of the equivalent amount of 50–80 % fraction should restore the full reducing power of the original juice, which it does (curve *D*, which is indistinguishable from *A*).

In the second set of experiments, cucumber juice was used as a source of the oxidase, as its content of reducing factor has been shown to be very low [Crook & Hopkins, 1938]. The oxidase was further purified by precipitation with 60 % saturated ammonium sulphate, re-solution and dialysis. The reductase was purified as above. Concentrations of the reactants are expressed as their equivalents of $N/100$ I_2 solution, and the experiments were carried out at pH 6 as by Hopkins & Morgan. Fig. 5 shows the effect of having much oxidase and relatively little of the reducing factor. The AA is somewhat protected, particularly at the beginning, and the GSH is not oxidized as fast as is ascorbic acid alone. Note too that, as is to be expected, the sum of AA and GSH disappearing in a given time is approximately equal to the amount of AA which disappears in the same time in the absence of GSH. In Fig. 6 the relative concentrations are reversed, there being now relatively more of the reducing system. The result is a typical protection curve, the concentration of AA remaining unchanged until all the GSH has been oxidized at a rate which is the same as that for AA alone.

The behaviour of the reducing factor with cysteine and thiolacetic acid was tested. The courses of the reactions are shown in Fig. 7, *A* and *B* being the chemical and catalysed reactions with GSH, *C* and *D* with cysteine, and *E* and *F* with thiolacetic acid. GSH is obviously far more efficacious than either of the others, whilst thiolacetic acid gives a 'catalysed' reaction which is not much larger than the chemical one.

DISCUSSION

That ascorbic oxidase and the reducing mechanism are separate entities seems to be beyond doubt. The fact that the reducing catalyst can be obtained in good yield virtually free from oxidase properties is the best evidence for this, but almost every other property is different. The Michaelis constant of the oxidase is of the order of a thousand times that of the reductase, the effect of inhibitors quite distinct and the thermolabilities different in the two cases. Finally, their relative activities in different samples of juice differ.

This being the case, it seems worth while to point out that Stewart & Stewart [1936] misquote Hopkins & Morgan when they ascribe the catalytic reduction of DHA to ascorbic *oxidase*, rather than to cauliflower press-juice. Similarly it is not difficult to see why Fujita & Numata [1938] were unable to repeat Hopkins & Morgan's results. They were using an oxidase preparation made according to Ebihara [1939], which would not be expected to contain much reducing catalyst; although they remark, 'if one adds too much oxidase, GSH is also oxidized'! It is surprising that they did not observe oxidation of GSH by DHA even chemically under the conditions used (pH 6 and 30°). Tauber [1938] too is wrong in stating that AA is not oxidized in the presence of GSH. The main point is that it can be reduced again faster than it is oxidized; but the O_2 uptake in the system is due to the oxidation of AA and not to the preferential oxidation of GSH.

In this connexion, it is interesting to note that Snow & Zilva [1938] report that dialysed cucumber juice, containing less than 0.1 mg. AA per 100 ml., is capable of oxidizing GSH and cysteine directly, whereas dialysed cauliflower juice is unable to do so unless a trace of ascorbic acid is introduced [Crook & Hopkins, 1938].

It would be as well to consider the evidence as to the enzymic nature of the reducing catalyst, for the activity could well be ascribed to a simple chemical substance acting as a cyclic catalyst, particularly as the increase in reaction velocity is seldom greater than a factor of 7 or 8 (although this, of course, might

be explained by its occurrence in very low concentrations). The evidence must be indirect, as no preparation pure enough for chemical investigation has been made. The chief evidence in favour of its being an enzyme is (i) non-diffusibility through a cellophane membrane, (ii) great thermolability, (iii) precipitation by acids and other 'protein reagents' [cf. Pfankuch, 1934], (iv) the occurrence of a pH-optimum. The last, rather than a general tendency of the curve, would hardly be expected for a simple substance.

The relation of the reducing mechanism here described to other protective mechanisms for AA which have been reported in plants is not clear. Barron *et al.* [1936] showed that in many plant juices AA remained unoxidized on exposure to air. At the same time they found that GSH protected AA against Cu-catalysed oxidation, a result also found by Hopkins & Morgan. The amount required was very small and equivalent to the Cu present, i.e. GSH acts here by fixing Cu as a non-catalytic complex. Damodaran & Nair [1936] isolated a tannin from the Indian gooseberry (*Phyllanthus emblica*) which would inhibit the oxidation of AA in the press-juice. They concluded that its action depended on suppression of heavy metal catalysis since the protection could be 'over-ridden' by massive amounts of Cu. Kardo-Syssayava & Nissenbaum [1938] report a thermolabile stabilizer for AA in the structural parts of the tomato, but give no indication of the mechanism concerned in its functioning. The same comment is true of the findings of Giri & Krishnamurthy [1940], who were able by treatment with acetone to separate the press-juice of certain plants into a fraction containing ascorbic oxidase and one containing a substance preventing the oxidation of AA, even in the presence of added Cu. Apparently, the effect of this inhibitor was to depress the O₂ uptake of the system generally, so that they are probably not dealing with the Hopkins-Morgan system. The claim of Giri & Krishnamurthy that, previous to their publication, there has been no indication in the literature of the coexistence of oxidizing and protective factors for AA in plants is hardly substantiated by the literature reviewed in this paper.

The possibility that the Hopkins-Morgan system or a modification thereof is one plant analogue of the cytochrome system in animals is probably worth considering. While not wishing to suggest that it is so, it may be worth while setting out certain observations in its favour. Several authors, notably Ganapathy [1938] and Lohman & Sanborn [1937], have shown that —S—S— is reduced in plant juices. The former has also shown that this effect is abolished by heating, i.e. depends on further enzyme systems. Further, Povolockaja [1937] found that the O₂ consumption of the AA ascorbic oxidase system fully accounts for that of the whole germinating seed in legumes and cereals. Thus the Hopkins-Morgan system could conceivably act as the last link in a chain:

The criticism by Barron [1939] of the validity of the work of Hopkins & Morgan and Crook & Hopkins on the grounds that both the ratio AA : GSH and the total concentrations used were unphysiological loses much of its weight in the light of the above considerations. In fact the concentrations of AA used by Hopkins and his collaborators are well within the ranges exhibited by plants and are much below that occurring in such plants as the black currant and Hungarian red pepper. Neither were their ratios unphysiological, since, according to the work of Pett [1936], GSH and AA occur in potatoes in a molar ratio of approximately 2 : 1.

More recent work, such as that of Ganapathy & Sastri [1938], who showed that papaya and similar fruits contain as much as 0.3 % of GSH, casts further doubt upon the validity of Barron's sweeping statement that 'there is very little glutathione in plants and a large amount of ascorbic acid'.

SUMMARY

1. The enzyme catalysing the reduction of dehydroascorbic acid by SH—glutathione has been separated from the associated ascorbic acid oxidase.
2. It is shown to be responsible for the protection of ascorbic acid by glutathione in cauliflower juice.
3. The fact that its activity varies in different plants has been mentioned.
4. Its properties and some preliminary observations on the kinetics of the reaction are described.
5. Its possible role as an 'end system' in plants is discussed.
6. Some indication is given that oxidation of ascorbic acid by the oxidase or by simple chemical reagents gives rise to the same form of dehydroascorbic acid.

The author wishes to express his indebtedness to Prof. Sir Frederick Gowland Hopkins for his continued helpful interest and gracious encouragement. He also wishes to thank Melbourne University for the award of the Australian Commonwealth Travelling Scholarship, during the tenure of which the experimental work in connexion with this and the previous paper was carried out.

REFERENCES

- Barron (1939). *Cold Spr. Harb. Symp.* **7**, 145, 146.
 — Barron & Klemperer (1936). *J. biol. Chem.* **116**, 563.
 Bersin, Köster & Jussatz (1935). *Hoppe-Seyl. Z.* **235**, 12.
 Birch, Harris & Ray (1933). *Biochem. J.* **27**, 590.
 Borsook, Davenport, Jeffreys & Warner (1937). *J. biol. Chem.* **117**, 237, 245, 269.
 de Caro & Giani (1934). *Hoppe-Seyl. Z.* **228**, 13.
 Crook & Hopkins (1938). *Biochem. J.* **32**, 1356.
 Damodaran & Nair (1936). *Biochem. J.* **30**, 1014.
 Ebihara (1939). *J. Biochem., Tokyo*, **29**, 199.
 Evelyn, Malloy & Rosen (1938). *J. biol. Chem.* **126**, 645.
 Fujita & Numata (1938). *Biochem. Z.* **299**, 249.
 Ganapathy (1938). *Curr. Sci.* **6**, 451.
 — & Sastri (1938). *Proc. Ind. Acad. Sci. B*, **8**, 399.
 Giri & Krishnamurthy (1940). *Nature, Lond.*, **146**, 99.
 Goldstein & Volkensohn (1938). *Biokhimiya*, **3**, 446.
 Golyanitskii & Belonosov (1936). *C.R. Acad. Sci. U.R.S.S. (N.S.)*, **4**, 15.
 Gudlet & Kardo-Syssayava (1938). *Biokhimiya*, **3**, 334.
 Herbert, Hirst, Percival, Reynolds & Smith (1933). *J. chem. Soc.* p. 1270.
 Hopkins & Slater (1935). *Biochem. J.* **29**, 2803.
 — & Morgan (1936). *Biochem. J.* **30**, 1446.
 Kardo-Syssayava & Nissenbaum (1938). *Biokhimiya*, **3**, 354.
 Kertesz (1938). *Biochem. J.* **32**, 621.
 Lohman & Sanborn (1937). *J. Industr. Engng Chem.* **20**, 189, 1195.
 Martin (1940). *Rep. 3rd Int. Congr. Microbiol.* p. 281.
 Mawson (1935). *Biochem. J.* **29**, 569.
 Moll & Wieters (1936). *Merck's Jber.* **50**, 65.
 Pett (1936). *Biochem. J.* **30**, 1228.
 Pfankuch (1934). *Naturwissenschaften*, **22**, 821.
 Povolockaja (1937). *C.R. Acad. Sci. U.R.S.S.* **17**, 35.
 Rubin, Sesakian & Lulikova (1937). *C.R. Acad. Sci. U.R.S.S.* **15**, 495.
 Snow & Zilva (1938). *Biochem. J.* **32**, 1926.
 Stewart & Stewart (1936). *Ann. Rep. Chem. Soc.* **33**, 389.
 Strachitskii & Rubin (1936). *Biokhimiya*, **1**, 642.
 Szent-Györgyi (1928). *Biochem. J.* **22**, 1387.
 — (1931). *J. biol. Chem.* **90**, 385.
 Tauber (1938). *Ergebn. Enzymforsch.* **7**, 308.