CCV. SOME RELATIONS BETWEEN ASCORBIC ACID AND GLUTATHIONE.

By FREDERICK GOWLAND HOPKINS and EDWARD JAMES MORGAN.

From the Biochemical Laboratory, Cambridge.

(*Received 30 June 1936.*)

Ascorbic acid and glutathione are the most conspicuous and, so far as is at present known, the most active reducing substances in living tissues. In spite of the fact that their fundamental constitutions and physiological functions are so different they have certain qualities in common. Both agree for instance in the circumstance that though their reduced and oxidized forms may co-exist in a tissue, they form redox systems which are not thermodynamically but only chemically reversible. Though in a given case the function of either may be specific, as is that of glutathione in the glyoxalase system, other systems are known in which one can replace the other, probably because in such cases reducing power alone determines their influence. Doubtless other more specific kinetic functions may be revealed in the future. Meanwhile the question arises whether, as reducing substances with different redox potentials, they can exert combined activities, or display interrelations of importance. This paper deals with their mutual relations as displayed in particular circumstances.

I. The system hexoxidase-ascorbic acid-glutathione *in vitro*.

Szent-Györgyi [1931] described a plant enzyme which oxidizes ascorbic (hexuronic) acid reversibly. He called it "hexoxidase" and though the name of its substrate has been changed it will be convenient, in this paper at least, to retain for the enzyme its original designation. Szent-Györgyi had already shown [1928] that ascorbic (hexuronic) acid was oxidized by metal catalysis but catalysis by the enzyme is much more efficient. In commenting on the specificity of the latter he remarked "also glutathione remains unoxidized in the presence of hexoxidase. If, however, hexuronic acid is present the glutathione is oxidized; the hexuronic acid plays the role of catalyst; it is oxidized by the enzyme and reduced by glutathione". Szent-Györgyi was therefore at that time conversant with the essential relations which hold in the system now to be described; but his studies went no further than what is expressed in the above qualitative statement. Meanwhile Mawson [1935] has studied the effect of tissue extracts in protecting ascorbic from oxidation and found that part, though by no means all, of their influence is due to their content of glutathione (GSH), a conclusion to which de Caro & Giani [1934] had also come. Kellie & Zilva [1934] showed that tissue extracts inhibit oxidation by copper. Bersin *et al.* [1935] have also shown that the autoxidation of ascorbic acid *in vitro* is inhibited by (in their experiments relatively large amounts of) GSH.

A closer and quantitative study of the system ascorbic acid-glutathione-enzyme has shown that it is one of much interest because of the definite manner in which it allows mutual relations to be displayed.

Methods.

Szent-Györgyi obtained the enzyme from the expressed juice of cabbage leaves. This source we have also used, but we have found the juice from the florets of the cauliflower and the central white stalks which carry them especially convenient, owing to its freedom from chlorophyll and to the much smaller amounts of soluble substances contained in it. The enzyme seems to be present in high concentration in all varieties of *Brassica*, and is certainly widely distributed in other species. To determine its distribution more exactly would be of interest. Zilva [1934] decided, doubtless rightly, that the rapid disappearance of ascorbic acid from apples was probably due to this enzyme. Tauber *et al.* [1935] prepared and studied an enzyme from *Curcubita maxima* which oxidizes ascorbic acid but which, though it would seem on insufficient grounds, they decided was different from Szent-Györgyi's hexoxidase.

Szent-Györgyi concentrated his enzyme preparation by precipitation with ammonium sulphate, and this method we have occasionally followed. In the majority of our experiments however we have used the expressed juice itself. If this contains oxidizing agents other than the hexoxidase they certainly do not exercise any influence on the system as studied. It was usually prepared from cauliflowers in bloom, being expressed from the plant tissues by the use of a screw press with a linen filter, and afterwards centrifuged. It is then clear, nearly colourless and contains a concentration of the enzyme which, though always high, varies somewhat with the source. It is always higher in the fresher home-grown plants than in those imported. In the present research it has not seemed necessary to standardize the strength of enzyme preparations used. In every case where the purpose of an experiment has made equality of concentrations necessary the same preparation has of course been used. In order that the relations to be discussed should be clearly shown it is necessary, as will immediately be understood, that the juice or enzyme preparation employed should be entirely free from ascorbic acid. 48 hours' dialysis in a cellophane dialysing tube with running water secures this freedom.

To follow the progress of concentration changes in the system when aerobic conditions were required solutions of the reactants were uniformly shaken in a series of open Erlenmeyer flasks, the contents of each flask being at first identical. At suitable intervals one of the flasks was removed, further change being immediately stopped by the addition of trichloroacetic acid, and the contents were titrated. The concentrations of ascorbic acid and glutathione (GSH) present at each stage of the experiment were determined by titrating half of the solution with $N/100$ iodine in the presence of potassium iodide and the other half with Tillman's reagent, using a micro-method [Birch *et al.*, 1933; Hopkins *et al.*, 1935]. The iodine value of the ascorbic acid so estimated was calculated and deducted from the total iodine figure to give the figure for glutathione. When these two substances are in solution alone, unassociated with other reducing substances, much experience has shown that this method gives results which are completely accurate.

Experimental results.

To save space all the results obtained are displayed in the figures without numerical tables to correspond. For convenience the concentrations of the ascorbic acid and glutathione are given in terms of ml. of $N/100$ iodine. This makes easier their presentation together in a single figure. It may be useful to

recall that to reduce a molecule of iodine two molecules of glutathione are required, but only one of ascorbic acid, and that 1 ml. of $N/100$ iodine corresponds to 3·07 mg. of the former and 0·88 mg. of the latter.

The oxidation of ascorbic acid itself as catalysed by the enzyme may be first illustrated. Fig. 1 shows the characteristic linear course of the reaction; its velocity being proportional to the enzyme concentration. Fig. 2 shows the marked effect of *p*H upon the velocity. It is much greater at *p*H 6·0 than at 7·4, the former being near to the *p*H of the expressed juice.

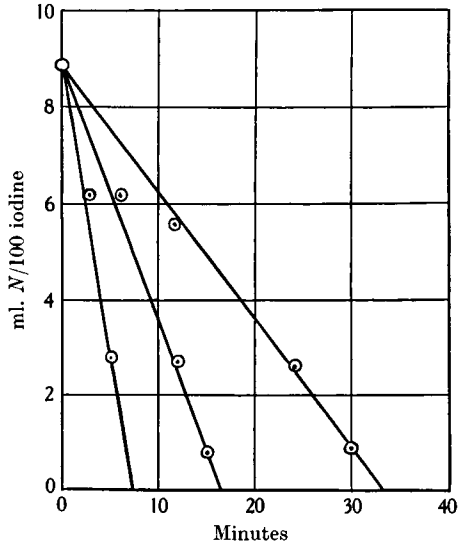

Fig. 1.

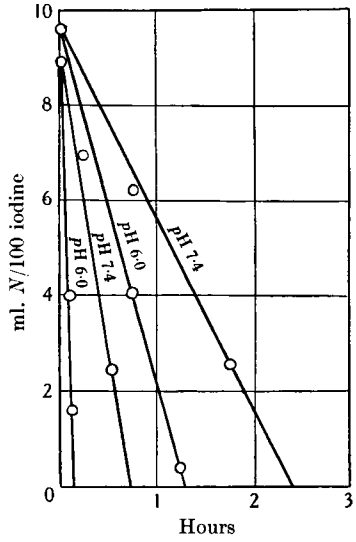

Fig. 2.

Fig. 1. Shows the course of oxidation of ascorbic acid (8 mg.) at *p*H 6·0 and 18° with 1, 2 and 4 ml. of the same enzyme preparation. The velocity is seen to be closely proportional to the enzyme concentration.

Fig. 2. Shows effect of *p*H on the velocity of oxidation by the enzyme in high and lower concentrations. Temp. 20°.

Fig. 3, which presents at a glance the relations which are always displayed in the system studied, has almost the appearance of a diagram, but is actually constructed from experimental data plotted in the ordinary way. In the experiments yielding the results which this figure summarizes the enzyme preparation was thoroughly dialysed juice and was identical and at the same concentration in each of the three experiments involved. They were carried out at *p*H 7·4, and the time scale extended in order that the relations should be clearly shown. The ascorbic acid and glutathione were present throughout in the proportion of approximately 1 mol. of the former to 2 of the latter. (If exactly in these proportions the resulting oxidation curves would overlap.)

The sloping dotted line shows the course of oxidation of ascorbic acid when alone with the enzyme. The horizontal continuous line shows the behaviour of glutathione when alone with the enzyme; it was unaffected. When however both substances were present together the ascorbic acid was wholly protected from oxidation (horizontal dotted line) whilst the glutathione was oxidized at exactly the same rate as was ascorbic acid when alone (sloping continuous line). It wil

be seen that when the glutathione was very nearly all oxidized, but only then, the oxidation of ascorbic acid promptly began. The completely linear course of both the direct oxidation of ascorbic acid and the indirect oxidation of glutathione is noteworthy, and also the circumstance that the courses are exactly parallel. These relations are always reproducible in the system, remaining essentially the same when the relative concentrations of the reactants are varied widely. Fig. 4 for instance shows the results of two experiments in which the enzyme concentration was higher than in those of Fig. 3 (the time scale being

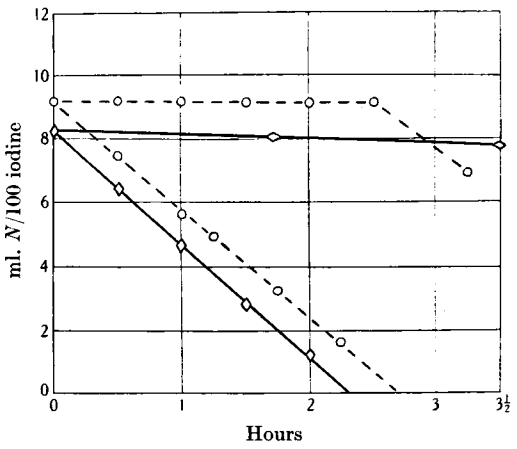

Fig. 3.

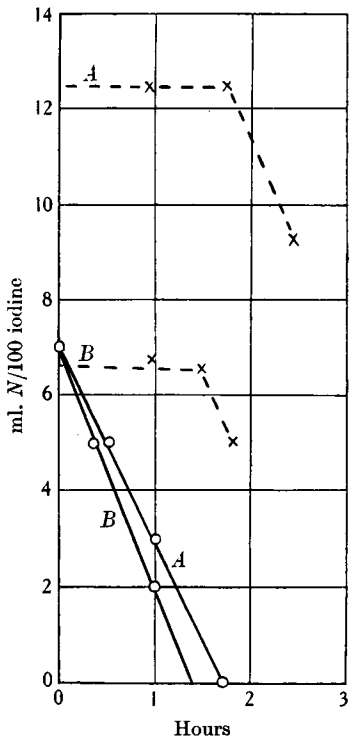

Fig. 4.

Fig. 3. Shows the influence of the oxidase in identical concentrations on ascorbic acid (broken lines) and glutathione (continuous lines), when alone and when together. In each case 1 ml. of dialysed juice in 10 ml. of solution buffered at *p*H 7·4. Temp. 18°. For discussion see text.

Fig. 4. Course of oxidations with both components initially present together. In case *A* the concentration of ascorbic acid was approximately twice that in *B*; that of GSH the same in each case. *p*H 7·4. Temp. 18°.

therefore shortened in the figure). In case *A* the concentration of ascorbic acid was twice that in case *B*, whilst the concentration of glutathione was the same. In each case it will be seen that the protection of the former lasted till the latter was nearly all oxidized.

The relations thus brought to light by the use of iodine titrations were further illustrated by measurements of oxygen uptake in the Barcroft differential manometer, the various adjustments in the instrument being made as usual.

Fig. 5 shows results which are typical of several obtained. A solution containing 2·64 mg. of ascorbic acid and 9·9 mg. of GSH (1 : 2 mol. approx.) buffered with phosphate to *p*H 6, was placed in a flask of the manometer; the total volume being 3 ml. In a Keilin cup 0·33 ml. of undialysed juice expressed from cauliflowers was placed, and this was tipped into the solution only after the apparatus was equilibrated in the bath (temp. 18°). Side by side with this a second manometer was shaken, containing, in precisely similar circumstances, 2·64 mg. of ascorbic acid alone. The juice was an active one and the *p*H near the optimum for the enzyme. The oxygen uptake was therefore rapid. It will be seen that its course was, as always, strictly linear, and its earlier stage, when the glutathione was alone being oxidized, continuous with the later stage of ascorbic acid oxidation. It will be also seen that the linear uptake ceased when the theoretical requirement for the oxidation of both constituents was only slightly exceeded. The oxidation rate of the ascorbic acid, when alone, corresponded so exactly with that of the two components together that the curves completely overlapped.

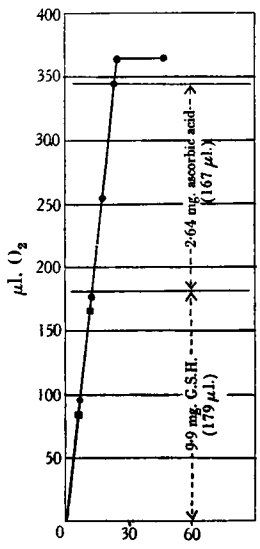

Fig. 5.

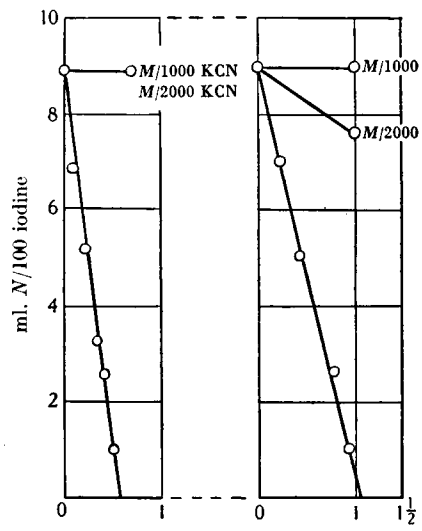

Fig. 6.

Fig. 5. Oxygen uptake of ascorbic acid and glutathione together in solution with the oxidase. The lower horizontal line shows the theoretical uptake for the GSH present and the upper that for both components together. The course of uptake for the ascorbic acid alone in identical conditions was separately determined and two points from the linear course are shown as squares. The velocity was exactly the same as in the case of the mixture.

Fig. 6. Inhibition of the enzyme by cyanide. The right-hand chart shows the effect in the case of the juice; inhibition was complete at *M*/1000 KCN. In the case of an enzyme preparation obtained by precipitation with $(NH)_4SO_4$ *M*/2000 was equally effective; left-hand chart.

Before discussing these results further a few words may be devoted to the nature of the enzyme. Szent-Györgyi found that it was relatively insensitive to cyanide inhibition, and on this circumstance, together with the fact that the rate of oxidation of ascorbic acid under its influence varied so slightly with varying concentrations of the substrate, he based the opinion that the mechanism of its action must be different from that of all other oxidases. We have not been

able to confirm the insensitivity to cyanide; $M/1000$ KCN we have found to inhibit oxidation by the juice completely (Fig. 6). In the case of an enzyme preparation obtained by precipitation with ammonium sulphate inhibition was complete at $M/2000$. The linear rate of oxidation, showing no loss of velocity with the fall in substrate concentration is not out of harmony with the Michaelis-Menten theory. In the case of the hexoxidase, however, it is somewhat remarkable that the velocity remains constant until the substrate is so nearly oxidized. Its kinetics would doubtless repay a thorough study. On the basis of the theory the Michaelis constant of the enzyme must be very small, and it is perhaps necessary to assume that it activates both the reduced and oxidized molecules of ascorbic acid. With regard to the nature of the oxidation suffered by the ascorbic acid molecule, although it has been suggested that this involves only the removal of two hydrogen atoms from the carbons which in the original molecule are united by a double bond, the classical work of the Birmingham School makes it almost certain that it involves rather the addition of two hydroxyl groups to those carbon atoms [Herbert *et al.* 1933]. The distinction however is perhaps unimportant as the adjacent carbonyl groups left by the removal of the two hydrogens would in any case almost certainly suffer hydration. The enzyme is quite unaffected by phenylurethane as we have found and, as Szent-Györgyi found, it does not accelerate the reduction of methylene blue by ascorbic acid. This and its cyanide-sensitiveness bring it under the accepted definition of an oxidase. Among oxidases it seems to be highly specialized in its activity, as Szent-Györgyi, and, since, Tauber *et al.* [1935] have found.

We have seen that the outstanding feature of the system formed when ascorbic acid and glutathione are together under the influence of hexoxidase is the complete protection from oxidation of the specific substrate and the indirect oxidation of the constituent which when alone is unaffected by the enzyme. There can be no doubt that this follows from the circumstance that hydrogen is transferred from two molecules of glutathione to each activated molecule of ascorbic acid, maintaining it thus in its reduced form. In explanation of the steady maintenance displayed so long as even very small amounts of glutathione remain in the system, it would seem that the oxidation of ascorbic acid should prove to be a slower process than its reduction. We have endeavoured to test this point experimentally.

As others have found, the product of the reversible oxidation of ascorbic acid is remarkably unstable and difficult to isolate. On the other hand solutions prepared by oxidation with iodine for instance, which was first used by Szent-Györgyi himself, have the disadvantage that when their pH is adjusted a relatively large amount of iodide must be present, making such solutions unsatisfactory for enzyme studies. To follow the rate of reduction by glutathione we proceeded therefore as follows.

Oxidation by the enzyme was first carried out by placing equal amounts of ascorbic acid in buffered solution at known pH, in each of a series of Thunberg tubes provided with stoppers carrying a bulb turned downwards. An equal amount of enzyme was measured into each of the tubes, and all, at this stage left open to the air, were uniformly shaken by a motor. The course of oxidation was followed as usual by removing individual tubes at intervals and determining therein the remaining concentration of reduced ascorbic acid. When reversible oxidation was just completed (*vide infra*) a known amount of glutathione in neutralized solution was measured into the bulbs and the tubes stoppered. All were fully evacuated and the glutathione then mixed with the main solution. The tubes were returned to the shaker and the increasing concentration of

reduced ascorbic acid followed by successive estimations. An advantage of this method of experiment is that each sample remains in the same vessel during the processes and the enzyme present is the same throughout. With regard to the preliminary oxidation it is important to realize that though the enzyme first oxidizes the ascorbic acid reversibly, if its action is allowed to continue long enough irreversible oxidation may follow. Fortunately however in solutions somewhat on the acid side of neutrality, e.g. at *p*H 6, the former process is completed before the latter begins. Moreover, since oxidation follows a linear course so exactly, it is easy after two successive estimations have been made in the series to follow the slope thus established down to the base line and thus learn the moment when the reversible oxidation will be complete but not exceeded. The information is thus given with remarkable accuracy. In the experiments under description the tubes were therefore removed for the reduction process just before the time so indicated, and the amount of reduced ascorbic acid ultimately restored in the reversal process was found to correspond, often exactly, with the amount present before the process of oxidation began.

The results of a typical experiment of the above sort are demonstrated in Fig. 7, in which it should be noted the time scale is in minutes, not hours. In

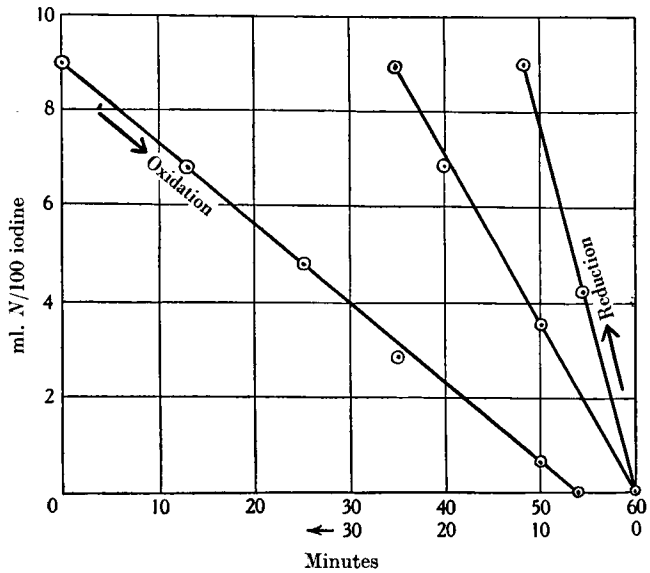

Fig. 7. Enzymic oxidation of ascorbic acid followed by reduction. The latter was induced by adding GSH to the system at the moment when reversible oxidation was complete. The right-hand line shows the rate of reduction in anaerobic conditions. It was five times as great as the rate of oxidation. In this experiment undialysed juice was used for catalysis (see text).

this experiment the reactants were employed in the proportion of approximately 1 mol. of ascorbic acid to 4 mol. of glutathione. When aeration began 7·8 mg. of ascorbic acid were present in each tube and when the oxidation was completed 60 mg. of glutathione were added for the reduction process. The solutions were buffered with phosphate buffer at *p*H 6, the total volume being in each flask 10 ml. during the oxidation and 12 ml. during reduction. The enzyme was that contained in 1 ml. of undialysed fresh juice. Temperature 21°.

In the figure the left-hand line shows the course of the preliminary oxidation of the ascorbic acid when alone, and that farthest to the right shows the (equally linear) course of the anaerobic reduction in the presence of the glutathione. It will be seen that the former occupied 58 min. and the latter (the original concentration of reduced ascorbic acid being exactly restored) 12 min., the velocity of the reduction at *p*H 6 being thus nearly five times that of the oxidation. The middle line of Fig. 7 shows merely the slower reduction which occurred when tubes containing the enzyme-oxidized ascorbic acid and the added glutathione stood open to the air without shaking.

These results were obtained, as stated, at *p*H 6 and have been frequently repeated. It was found difficult to reproduce them satisfactorily at 7·4, for some at least of the irreversible product then appears early in the course of oxidation. That, in the absence of the enzyme, slow irreversible oxidation by molecular oxygen occurs at *p*H 7·4 and upwards is well known [Barron *et al.* 1936].

A variation in experiments of the kind last described may be looked upon as supplementing those illustrated in Figs. 3 and 4. The ascorbic acid was first oxidized by the enzyme as before, but the subsequent reduction by GSH, instead of being anaerobic, was allowed to proceed while shaking the solution in open flasks was continued. The results of one such experiment may be briefly reported.

In this the concentration of the enzyme was made relatively high (2 ml. of active juice in 10 ml. of buffered solution at *p*H 6). At the beginning of the experiment 7·85 mg. of ascorbic acid were present. The reversible oxidation of this took 18 min. for completion. Neutralized GSH in excess (120 mg. in 2 ml. water) was then added, and aeration continued for exactly 18 min. to correspond with the oxidation period; further change was then stopped by the addition of trichloroacetic acid. The oxidation of ascorbic acid during the second period was balanced by reduction and at the end it was fully reduced. Of glutathione 57·3 mg. were found to have been oxidized, while for the reduction of 15·7 ($=7{\cdot}85\times2$) mg. of ascorbic acid 54·7 mg. are required. As the glutathione preparation may have been to some slight degree autoxidizable, the correspondence is satisfactory. In this case as in experiments of Figs. 3 and 4 the rate of reduction of ascorbic acid, and therefore that of the oxidation of GSH, was of course controlled by the slower reaction of the oxidation of the former.

In the above experiments relatively high concentrations of GSH were employed. With lower concentrations in the same experimental conditions the velocity of reduction becomes less but is always much higher than the oxidation rate. When the concentration is made very low only the initial velocity can be determined as the GSH is of course soon all oxidized.

One important comment remains to be made on the reduction process as it occurs in the system studied. The experiments just described were carried out with fresh juice undialysed. In an experiment carried out with an enzyme preparation made by precipitating the fresh juice with ammonium sulphate reduction also proceeded normally under its influence. On the other hand, an unexplained effect of dialysing the juice itself calls for discussion. In no other type of experiment have we found any difference between the activities of dialysed and undialysed juice. It should be recalled that in those of Figs. 3 and 4, which first revealed the essential relations in the system, dialysed juice was used. These, of course, were aerobic throughout; and it would seem sure that the reduction by GSH which in such conditions maintains the ascorbic acid in the reduced form must be a relatively rapid process. Yet in experiments of the kind

last described, in which the oxidation and reduction of ascorbic acid were made separate and successive processes, undialysed and dialysed juices are found to behave differently, though the experiments were carried out on identical lines in each case. Under the influence of the fresh juice the reduction, as we have seen, proceeds rapidly in linear fashion to completion; with the dialysed this is not the case. With the latter the reduction is found to have a high initial velocity but to fall off quickly, ultimately becoming linear at a rate which, instead of being much greater, is less than the rate of oxidation as observed in the first phase of the experiment. This effect of dialysis is not accidental; it has been obtained with juices from different sources all of which induced the rapid reduction when undialysed. It is noteworthy that the curves obtained after dialysis, like those obtained with the fresh juice, were closely similar in all cases though so different from the latter. It should be noted too that the velocity with which any particular specimen of juice oxidizes the ascorbic acid is entirely unaffected by dialysis; the result is therefore certainly not due simply to a falling off in the concentration of the enzyme. The change, moreover, does not occur if a preparation is allowed to stand untreated for as long or longer than the time taken for dialysis. If the removal of a co-enzyme or any equivalent factor were involved, the facts would be of interest as suggesting a difference in the activating mechanisms for oxidation and reduction respectively. We have not been able, however, to re-establish the original activity by adding the dialysate to the dialysed juice. There is again no factor in the undialysed juice which activates glutathione in the sense of increasing its power to reduce methylene blue. At present we have found no explanation for this unexpected effect of dialysis. To discover some method for preparing the oxidase in a form as pure as possible must be the first step towards such an explanation.

It may be noted here that the reduction of ascorbic acid by GSH when uncatalysed is a slow process. One experiment in illustration of this may receive mention. Ascorbic acid was oxidized by quantitative treatment with $N/10$ iodine in potassium iodide and the iodine then removed as lead iodide. The slight excess of lead was removed by adding potassium sulphate and the solution finally adjusted to *p*H 6. Using this solution the reduction by GSH alone was then followed by the same method as that used in previous experiments (e.g. in that yielding the results summarized in Fig. 7). It was found that in 90 min. only 3·5 mg. of ascorbic acid were reduced whereas in a control experiment, exactly similar, except for the presence of a low concentration of enzyme in the solution, the above amount was reduced in 8 min., and 6·1 mg. in 15 min.

II. The behaviour of ascorbic acid and glutathione in catalysis by copper.

Before discussing this it may be recalled that Barron *et al.* [1936] in a careful study of ascorbic acid oxidations showed that it is not autoxidizable at any *p*H below 7·6, whereas at *p*H 5·15 for instance its oxidation is actively catalysed by minute amounts of copper. As is well known Meldrum & Dixon [1930] found that pure glutathione is not autoxidizable and is only oxidized by metals when they are in association with an organic factor. They found this to be true of copper as well as of iron. Voegtlin *et al.* [1931] claim on the other hand that, unlike iron, copper itself is a powerful catalyst at any *p*H which is physiological. Our own experience agrees with that of the former authors though we have found it somewhat more difficult at, say, *p*H 7·4 to obtain preparations quite as stable to copper as to iron. At *p*H 6 the same preparations may be

stable. In any case the addition of a minute amount of material from a sample of glutathione which has undergone slight decomposition greatly increases the activity of the copper [cf. Meldrum & Dixon, 1930].

In Fig. 8 are summarized the results of an experiment showing the effect of copper upon the oxidation of glutathione and ascorbic acid when separate and when together in solution at *p*H 7·4. The technique of shaking, etc., was the same as in the enzyme experiments. The same concentration of the metal was present in each of the three observations involved. It was added as cupric chloride equivalent to 0·018 mg. of Cu in the 10 ml. of solution employed. The ascorbic acid (4·2 mg.) when alone was rapidly oxidized (lowest line) and the GSH alone (28 mg.) much more slowly (upper continuous line). When both were in solution

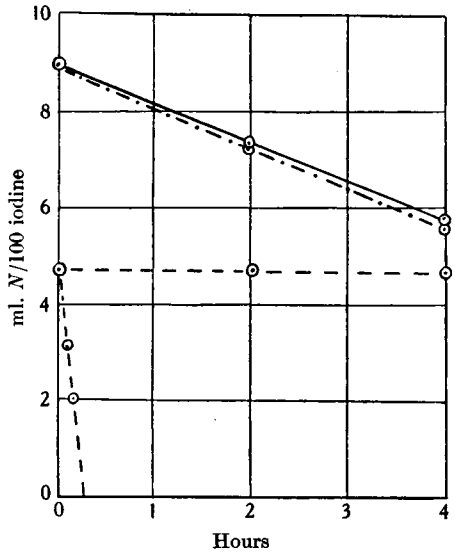

Fig. 8. Behaviour of ascorbic acid and GSH, when alone or together, during catalysis by copper. The lower sloping line shows the initial velocity of ascorbic acid when alone, the horizontal line shows its complete protection in the presence of GSH. The upper lines show the course of GSH oxidation with and without the presence of ascorbic acid. *p*H in each case 7·4. Cu 0·018 mg. in 10 ml. of solution buffered with phosphate.

the ascorbic acid was wholly protected from oxidation (horizontal broken line) its presence however having no effect upon the rate of oxidation of the GSH (upper broken line). This experiment was done with a sample of glutathione which, though stable in the absence of copper, had not been stabilized by Meldrum & Dixon's method. After it had been treated in solution with a large proportion of muscle powder on the lines described by these authors the experiment just described was repeated. The results however were so alike that the curves could be almost superimposed.

Fig. 9 refers to an experiment done on precisely similar lines but at *p*H 6. At this *p*H the sample of glutathione employed was stable. The total iodine used in titration remained constant throughout, neither constituent being oxidized (horizontal line). Such a system is inert; one which, as other experiments have shown, takes up no oxygen at all when shaken in a Barcroft manometer.

Clearly, the protection of ascorbic acid by glutathione when the catalyst is a metal must depend upon relations differing entirely from those which secure

protection in catalysis by the enzyme. Here there is no question of hydrogen transfer. When the glutathione itself is oxidized in the system the presence of ascorbic acid has no effect upon the velocity of its oxidation (Fig. 10). When, as at *p*H 6, the glutathione is not oxidized the system as just seen is inert. The protection must be due to the fact that the presence of GSH inhibits the influence of the metal on ascorbic acid—a more common type of catalysis inhibition. It must be supposed that although, unlike ascorbic acid, it is itself not oxidized by copper in the absence of an associated organic factor, the thiol form of

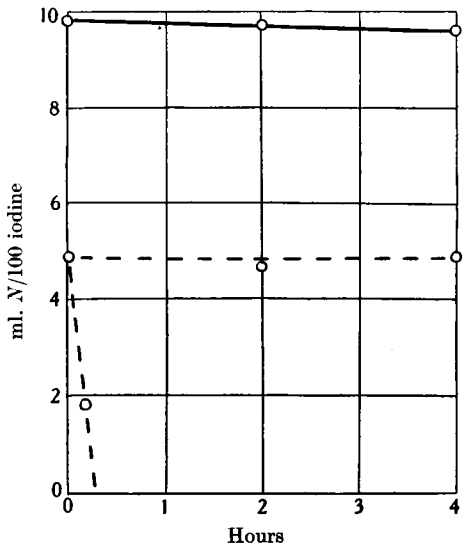

Fig. 9.

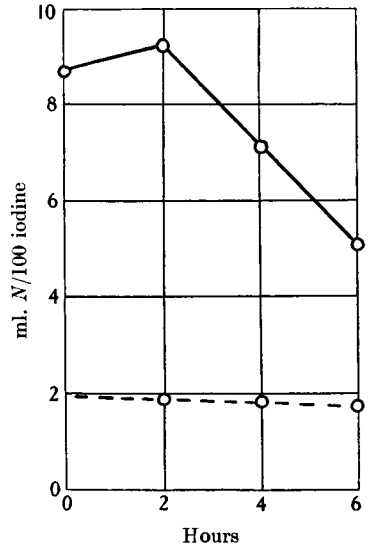

Fig. 10.

Fig. 9. Shows results of an experiment similar in all respects to that of Fig. 8 but carried out at *p*H 6. At this *p*H the GSH was stable and the system with ascorbic acid and enzyme was wholly inert.

Fig. 10. Course of oxidation of GSH (continuous line) and ascorbic acid (dotted line) during the aeration of liver tissue from well-fed rabbits.

glutathione (GSH) has a higher affinity for the metal than has ascorbic acid, and prevents effective contact between it and the latter. As might be expected our experiments have shown that the reduction of ascorbic acid by GSH is not catalysed by copper.

III. Some relations in hepatic tissue.

Before dealing with certain oxidation relations between ascorbic acid and glutathione in liver tissue brief reference may be allowed to experiments made six years ago [Hopkins & Elliott, 1931]. These showed that when liver pulp, suspended in mammalian Ringer solution, is aerated by shaking, the course of the oxidation of GSH exhibits characteristically a preliminary period during which reducing processes continue, maintaining or even increasing in fully aerobic conditions the concentration of the GSH. These experiments were made before the importance of ascorbic acid as a reductant was recognized and iodine titration was relied upon for the results obtained. Since then these experiments

have been many times repeated, the iodine value of the ascorbic acid present being deducted from the total iodine and the glutathione values obtained by difference plotted as before. The resulting curves display precisely the same form as those published in the paper just quoted.

In other experiments liver slices have been used yielding curves exactly similar in kind. As bearing on the accuracy of such determinations we have found that if hexoxidase be added to a protein-free aqueous extract of the liver or of most other animal tissues it very rapidly reduces the iodine titration figure of the extract to zero. Having regard to the specificity of the action of the enzyme this seems strong evidence in favour of the claim that only ascorbic acid and glutathione are titrated.

Some two years ago we made a number of experiments in which the oxidation rates of glutathione and ascorbic acid in aerated liver tissue were recorded simultaneously. The livers of rabbits in different nutritional conditions were employed. There is at present no evidence that animal tissues contain any enzymic catalyst for the oxidation of either substance. There is no doubt on the other hand that the liver contains sufficient copper to oxidize the ascorbic acid present at a much greater velocity than is observed in the excised tissue, and the intention of the experiments was to discover whether the presence of glutathione plays any part in protecting it from oxidation.

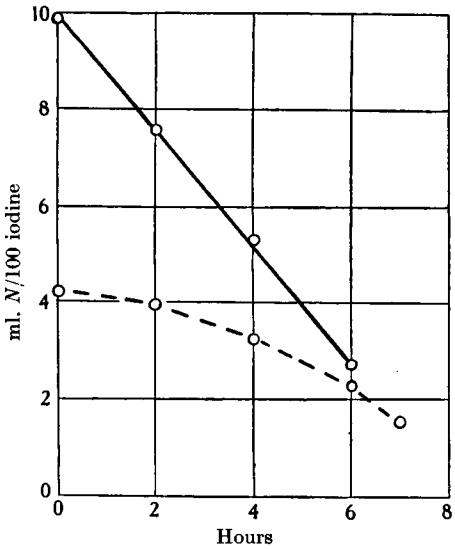

Fig. 11.

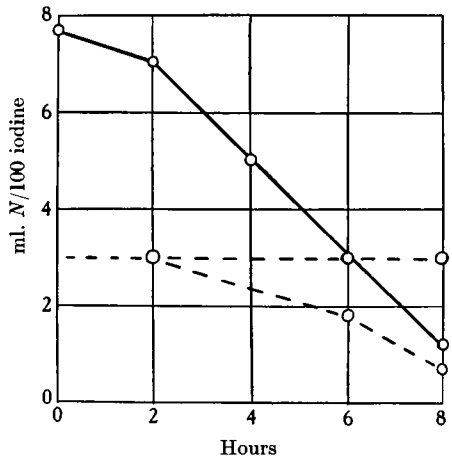

Fig. 12.

Fig. 11. Results of an experiment similar to that of Fig. 10 but obtained from the liver of a rabbit previously deprived of food for 48 hours.

Fig. 12. From an experiment similar to those of Figs. 10 and 11, but showing that ascorbic acid is protected from oxidation if the concentration of GSH is kept high throughout the aeration.

In Figs. 10 and 11 are curves selected from many obtained in the unpublished experiments under reference. In the first the preliminary period of sustained reduction of GSH was well marked and its oxidation relatively slow. The concentration of ascorbic acid is seen to have remained constant. The second shows other conditions. This experiment was done on the liver of a rabbit which had

been deprived of food for 48 hours; there was no reduction period, and the oxidation of GSH was rapid. In this case the ascorbic acid was also oxidized with some rapidity. In a general sense the other experiments in the series gave similar indications. Whilst, however, the two curves presented might seem to suggest that the same influences—identical reducing processes for instance—are responsible for controlling the rate of oxidation of both substances, other experiments of the same kind showed that there is too much irregularity in the results for this to be likely. With regard to any possible protective influence on the part of glutathione the curves obtained showed that any observed arrest or slowing of the oxidation of ascorbic acid is not related in any definite way to the absolute or relative concentration of the former existing at the time. If, it is true, the concentration of GSH be maintained at a relatively high value by adding it to the suspension of liver tissue during the course of the oxidations the ascorbic acid may be wholly protected. This is illustrated by the experiment of Fig. 12. Such artificial additions, however, do not reproduce the conditions present in the liver cell itself. It must be admitted that the experiments did not throw any clear light upon the relations of the two substances in the cell. They yield a suggestion that the tripeptide affords some protection to the vitamin but a definite proof or disproof of this calls for experiments of a different kind. The results obtained seem nevertheless to be worthy of record.

Though not strictly germane to this paper some further reference may be here allowed to facts concerning the oxidation of glutathione itself; facts bearing on the question as to how far the substance functions in the transport of hydrogen to oxygen. Experiments are in progress to determine the nature of the reducing systems which, as the experiments of Hopkins & Elliott [1931] showed, survive in excised tissues and, in spite of full aeration of these, delay for a period the oxidation of the GSH. This work is not yet complete and the results will be reported later. It may be stated, however, that the hydrogen donators mainly involved and probably the enzyme or enzymes are special in kind. Mann [1932] showed that the glucose dehydrogenase of Harrison can reduce the peptide, and Meldrum & Tarr [1935] that the isolated dehydrogenase system of Warburg & Christian can do so, even under aerobic conditions. If these systems are both active in the liver (the former certainly is) they are not the sole or most active systems concerned.

We wish, however, to comment here more particularly on a point concerned with the oxidation of GSH. Ogston & Green [1935] found that though rapidly reduced by the glucose and hexosemonophosphate dehydrogenases glutathione does not, when added to these in the presence of their substrates, increase the rate of oxygen uptake; the limiting factor being the rate of its oxidation when reduced. We have found, however, as others have found to be the case with ascorbic acid, that certain factors contained in tissue extracts strongly inhibit even in very low concentration the oxidation of "active" preparations of glutathione; preparations for instance which have stood long enough in aqueous solution to develop the organic factor which Meldrum & Dixon showed to be necessary (together with traces of metal) for oxidation. We have separated from liver a fraction containing a soluble protein (which may have been only an associate of some active inhibitor) of which 5 mg. when added to 25 mg. of activated glutathione in 10 ml. of solution at *p*H 7·4 completely inhibited its oxidation. We have moreover made preparations on orthodox lines of some dehydrogenases and have found that these may contain potent inhibitors of the kind in question. It is necessary therefore to bear these facts in mind in testing the capacity of glutathione to transfer hydrogen from a given dehydrogenase

system to oxygen. In our study of this question we have endeavoured to obtain cell-free extracts from the liver capable of oxidizing GSH. It is clear that such extracts while containing the oxidizing agency must be free from the inhibitory factors just mentioned. The following simple and empirical procedure yields preparations which are active. The liver, thoroughly ground up with sand, is extracted with mammalian Ringer solution, maintained at *p*H about 4·5 during the extraction by adding acetic or hydrochloric acid. It is so extracted three or four times and the mixed extracts centrifuged. The supernatant fluid is brought exactly to *p*H 4·5 and then heated to 70–75° and held at that temperature for 3–4 min. It is cooled and the coagulated proteins separated on the centrifuge. The oxidation factors in the extract are not enzymic and it may, if necessary, be concentrated *in vacuo* at low temperatures; not however open on a water-bath. At the above *p*H and in the presence of the electrolytes in the Ringer solution the inhibitory substances are removed with the bulk of the proteins while the oxidizing factors are left largely intact.

The two curves of Fig. 13 show the course of oxidation of GSH when an extract containing it was shaken in a series of open flasks. Oxidation was slow

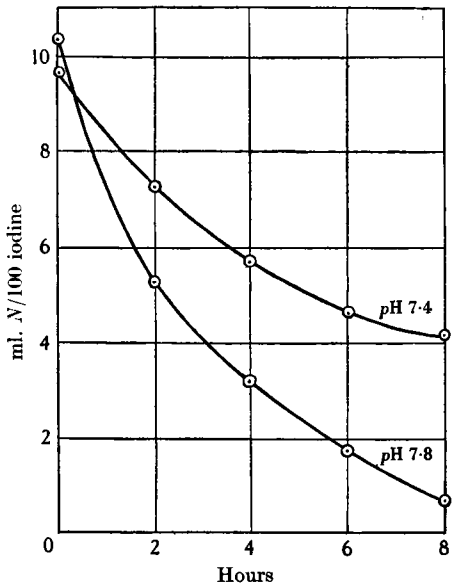

Fig. 13. Oxidation of GSH by cell-free liver extracts at *p*H 7·4 and 7·8. Temp. 18°.

but the experiments were done at room temperature and not at 37°. The characteristic linear course always seen when the liver tissue itself is aerated is not maintained in extracts and must depend on organizing conditions in the cell.

Discussion.

In 1928 Szent-Györgyi when describing for the first time the plant enzyme which specifically catalyses the oxidation of ascorbic acid, pointed out that although the enzyme when alone is without influence on reduced glutathione it oxidizes this when ascorbic acid is present in the system. The latter then "plays the role of a catalyst".

The experiments described in the first section of this paper dealt quantitatively with the system glutathione-ascorbic acid-enzyme. They have shown that so long as it remains in the system GSH completely prevents the oxidation of ascorbic acid. On the other hand, it is itself then oxidized at exactly the same rate as, in similar conditions, ascorbic acid is oxidized when alone. In each case the course of oxidation is linear throughout. Whatever the initial relative concentrations of the components that of ascorbic acid remains constant till the GSH is nearly completely oxidized. It seems clear that during the course of oxidation in the complete system the two hydrogen atoms which, in effect, are transferred from each activated ascorbic acid molecule to oxygen are simultaneously replaced by hydrogen from two molecules of GSH. The rates both of the direct and indirect oxidations are proportional to the enzyme concentrations and are affected alike by variations in *p*H.

The behaviour of the enzyme accords with the definition of an oxidase. It is fully cyanide-sensitive but unaffected by phenylurethane; its presence does not affect the rate at which methylene blue is reduced by ascorbic acid or glutathione. The circumstance that the course of the oxidation of ascorbic acid as catalysed by the enzyme remains linear throughout indicates that it has a high affinity for its specific substrate and that the Michaelis constant must in its case be very small.

To explain the steady maintenance of the reduced form of ascorbic acid in the presence of the oxidase when GSH is also present it seems necessary to assume that reduction of the former by the latter must be a more rapid process than the direct enzymic oxidation. It proved easy to show experimentally that this is the case if the enzyme is supplied as it exists in the undialysed plant juice or when it is contained in preparations made by precipitation from the juice with ammonium sulphate. Ascorbic acid, first oxidized reversibly by the juice or by such preparations, is reduced anaerobically when GSH is then added to the system at a rate which may be five times that of the oxidation. It is a remarkable circumstance however that, though the rate of oxidation induced by any sample of juice is not at all affected by dialysis, the power of the latter to catalyse reduction is much lessened and the course of reduction modified. This phenomenon which is consistently observed is discussed in section I, but no explanation can at present be offered. This, we think, is a reason for a further thorough study of the kinetics of the system. There is perhaps another. If we assume that the oxidized no less than the reduced molecules of ascorbic acid are activated by the enzyme it would seem unnecessary to suppose that the latter exercises any direct influence on the molecules of glutathione. Yet their reducing influence continues unabated when their concentration has become extraordinarily small. It may be justifiable to suppose that they are specifically absorbed and orientated on the enzyme surface and so brought into effective relations with the ascorbic acid molecules although not themselves activated.

The system as studied is an artificial one though its kinetics seem to be of great interest. In any plant tissue which contains glutathione however it might well have physiological functions. Though the presence of glutathione in the growing tissues of *Brassica* is not disproved, we have evidence that it is unlikely, though substances yielding a nitroprusside reaction are present.

The addition of a very minute concentration of ascorbic acid will convert a hexoxidase preparation which is without action on GSH into one which oxidises it, and it is perhaps instructive to realize that if, in the absence of Szent-Györgyi's recognition of his oxidase as one specific for ascorbic acid, a search had been made for an enzyme acting on GSH, it might well have been

supposedly found in the juice of *Brassica*. This would have proved to be inactive after purification; a co-enzyme would almost certainly have been then sought and identified with ascorbic acid!

The presence of GSH protects the vitamin from oxidation by copper catalysis no less than by enzymic catalysis. In the former case however the nature of its influence must be wholly different. There can be no question of hydrogen transference. If the glutathione employed in an experiment (owing to its association with small amounts of Meldrum & Dixon's organic factor) is to any degree oxidized by the metal, the presence of ascorbic acid makes no difference to the rate of its oxidation. If, on the other hand, the glutathione is stable the system is completely inert.

The inhibition of the ascorbic acid oxidation seems clearly to be due to a circumstance familiar in other cases of metallic catalysis. The inhibitor—in this case GSH—forms a stable compound with the copper, preventing efficient contact between the metal and its substrate.

It is of interest to find that one cell constituent protects another from oxidation in each of two systems so widely diverse in their mechanisms.

In section III of the paper reference is made to a repetition of the experiments of Hopkins & Elliott [1931] on the oxidation of glutathione in excised liver tissue. The results of these have been confirmed. Other experiments are described in which the oxidation rates of glutathione and ascorbic acid were followed simultaneously. Although they suggest that high concentrations of the former may protect the latter from oxidation in the liver, their general indications are that normally the two substances are oxidized independently, perhaps by different agencies.

Summary.

When ascorbic acid and glutathione are together in the presence of the hexoxidase described by Szent-Györgyi the glutathione wholly protects the vitamin from oxidation, whilst it is itself oxidized at a rate which, with the same concentration of enzyme, is exactly the same as the rate with which ascorbic acid is oxidized when alone. Only when GSH has practically disappeared from the system does the oxidation of ascorbic acid begin.

When ascorbic acid has been reversibly oxidized its reduction by pure glutathione alone is a very slow process; but in the presence of the enzyme (in conditions which are discussed in section I) the reduction may be five times as fast as the oxidation induced by the same concentration of the enzyme.

Glutathione also completely protects ascorbic acid from oxidation by copper catalysis. The mechanism of protection must here be different from that which operates in the case of the enzyme. In the latter it depends upon hydrogen transference, in the former on inhibition of the catalysis.

In the last section of the paper the behaviours of ascorbic acid and glutathione in aerated hepatic tissue are described and discussed.

REFERENCES.

Barron, de Meio & Klemperer (1936). *J. biol. Chem.* **112**, 625.
Bersin, Koster & Zusatz (1935). *Hoppe-Seyl. Z.* **235**, 12.
Birch, Harris & Ray (1933). *Biochem. J.* **27**, 590.
de Caro & Giani (1934). *Hoppe-Seyl. Z.* **228**, 13.
Herbert, Hirst, Percival, Reynolds & Lamb (1933). *J. chem. Soc.* p. 270.
Hopkins, Slater & Millikan (1935). *Biochem. J.* **29**, 2803.
—— & Elliott (1931). *Proc. roy. Soc.* B **109**, 58.
Kellie & Zilva (1934). *Biochem. J.* **28**, 661.
Mann (1932). *Biochem. J.* **26**, 785.
Mawson (1935). *Biochem. J.* **29**, 1028.
Meldrum & Dixon (1930). *Biochem. J.* **24**, 472.
—— & Tarr (1935). *Biochem. J.* **29**, 108.
Ogston & Green (1935). *Biochem. J.* **29**, 1983.
Szent-Györgyi (1928). *Biochem. J.* **22**, 1387.
—— (1931). *J. biol. Chem.* **90**, 385.
Tauber, Kleiner & Mishkind (1935). *J. Biochem., Tokyo*, **110**, 211.
Voegtlin, Johnson & Rosenthal (1931). *J. biol. Chem.* **93**, 435.
Zilva (1934). *Biochem. J.* **28**, 661.