THE REDUCTION AND OXIDATION OF GLUTATHIONE BY PLANT MITOCHONDRIA [1,2]

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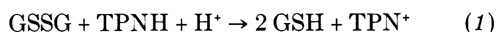
The reduction of oxidized glutathione (GSSG) by reduced triphosphopyridine nucleotide [3] (reaction *1*)



$$GSSG + TPNH + H^+ \rightarrow 2\,GSH + TPN^+ \qquad (1)$$

is catalyzed by glutathione reductase (3, 4, 5). In earlier studies this reaction has been coupled with soluble dehydrogenases which utilize TPN as a coenzyme (6). Since tissue fractionation studies have shown that numerous dehydrogenases, including those associated with the oxidation of the Krebs cycle intermediates, are localized within the mitochondrion, it was of interest to determine whether these enzymes might also be coupled with reaction *1*. This paper reports that it is possible to demonstrate the reduction of GSSG by plant mitochondria in the presence of suitable substrates. In addition, the plant particles also catalyzed the oxidation of GSH by molecular O_2 in the presence of catalytic amounts of ascorbic acid.

METHODS AND MATERIALS

The avocado mitochondria were obtained from firm fruit (Fuerte and Nabal varieties) purchased in the local market. The isolation procedure employed was that of Biale and Young (7). The mitochondria obtained in this manner were washed by suspending in approximately 150 ml of 0.5 M sucrose and recentrifuging at 17,000 × g. The particles were then suspended in approximately 5 ml of 0.5 M sucrose to make a suspension containing 1 to 2 mg N per ml. Pea mitochondria were obtained from 6- to 12-day-old etiolated pea seedlings (Alaska variety) or from the epicotyls of such plants. The prechilled plant material was ground with 300 ml of 0.5 M sucrose in a cold mortar with coarse, acid-washed sand. The pH of the homogenate was adjusted to 7.0 during grinding and the particles were isolated and washed in the same manner as for the avocado mitochondria. All manipulations were performed at 0 to 4° C and the mitochondria were used immediately after preparation. The nitrogen content of the mitochondria was determined according to Ma and Zuazaga (8). The GSSG reductase analysis is described elsewhere (4).

The reactions were carried out in the Warburg apparatus at 25° C. In the initial experiments all reagents were added to the main compartment of the flask and the mitochondria were placed in the side arm. Ten minutes were allowed for equilibration before tipping in the mitochondria from the side arm to initiate the reaction. In the anaerobic experiments, the vessels were flushed with nitrogen for 5 minutes before equilibration. At the end of the experimental period (60 to 85 minutes) 0.5 ml of 20 % metaphosphoric acid was added and the precipitate centrifuged down and discarded. The supernatant fraction was then analyzed for GSH according to the method of Fujita and Numata (9). The results obtained are expressed as micromoles of GSSG reduced (micromoles of GSH formed divided by 2).

In the experiments on oxidative phosphorylation the above procedure was modified by adding the mitochondria directly to the reaction mixture and placing radioactive inorganic phosphate ($P^{32}O_4^{\equiv}$) in the side arm. After flushing the anaerobic vessels with nitrogen and allowing all vessels to equilibrate for ten minutes, initial readings were taken. The $P^{32}O_4^{\equiv}$ was tipped in from the side arm at this time. After somewhat shorter experimental periods (30 to 40 minutes) the reactions were stopped with 0.5 ml 35 % trichloroacetic acid. The precipitated proteins were centrifuged off and aliquots of the supernatant fraction were analyzed for GSH and inorganic phosphate (10). The remainder of the supernatant fraction was analyzed for esterified P^{32} according to the method of Lehninger (11).

TPN^+ of 70 % purity was generously provided by Dr. M. A. Mitz of Armour Laboratories. DPN^+, AMP-5 and ATP were purchased from Pabst Laboratories; GSSG and GSH from Schwarz, Inc. The $P^{32}O_4^{\equiv}$ obtained from the Atomic Energy Commission was hydrolyzed in dilute acid before use (12).

RESULTS

The oxidation of Krebs cycle intermediates by mitochondria from avocado fruit was first reported by Millerd et al (13). In subsequent studies, Biale and his associates have extensively investigated the enzymatic properties of mitochondria from avocado fruit at all stages of the ripening process, and in particular have determined the optimum conditions for oxidative phosphorylation by these particles (7, 14, 15). With avocado mitochondria as a source of the necessary enzymes, it is also possible to couple the oxidation of the Krebs cycle acids to the reduction of GSSG when GSSG is substituted for O_2 as the oxidizing agent. Table I shows that the oxidation of citrate, succinate, malate and α-ketoglutarate can be carried out with either oxidant. The first column gives the O_2 uptake observed with the particles in air in the absence of GSSG. The second column shows the

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[3] The following abbreviations have been used: TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotide; DPN^+ and DPNH, oxidized and reduced diphosphopyridine nucleotide; GSSG and GSH, oxidized and reduced glutathione; AMP-5, adenosine-5′-PO_4; ATP, adenosine triphosphate.

TABLE I

REDUCTION OF OXIDIZED GLUTATHIONE BY KREBS CYCLE INTERMEDIATES

CONDITIONS	O_2 UPTAKE MICRO-ATOMS/MG N × HR	GSSG REDUCED MICRO-MOLES/MG N × HR
No substrate	0.7	0.08
Citrate	4.2	3.15
Succinate	12.1	2.28
Malate	2.6	2.01
α-Ketoglutarate ...	10.1	2.59

Reaction mixtures contained 0.02 M substrate; 1.5×10^{-3} M AMP-5; 10^{-3} M $MgSO_4$; 4.5×10^{-3} M GSSG; 6×10^{-5} M TPN^+; 0.01 M phosphate buffer, pH 7.1; 0.3 M sucrose; and 1 × washed avocado particles containing 1.01 mg N. Total volume, 2.2 ml. Center well contained 0.1 ml 20 % KOH. Gas phase, air or N_2. Reaction time, 80 min.

amount of GSSG reduced when GSSG was added to the reaction mixture and the reaction was carried out in N_2. Since the reduction of one atom of oxygen to H_2O requires two electrons, this reaction is equivalent to the reduction of one mole of GSSG to GSH. The values given in table I and subsequent tables are therefore directly comparable. Although GSSG was readily reduced, it is apparent that the amount of O_2 consumed was generally greater than the amount of GSSG reduced. The variation in O_2 consumption with the different substrates was also greater than the variation in the amount of GSSG reduced and suggests that the reduction of GSSG may be a rate-limiting reaction.

The reduction of GSSG by citric acid was selected for a more detailed study. In these experiments, GSSG was added to incubation mixtures containing the necessary components for the oxidation of citric acid (AMP-5, Mg^{++}, citrate, and mitochondria) and the reactions were carried out in N_2 and in air. As shown in table II, GSSG was reduced both in N_2 and in air. The reaction in both instances was clearly dependent upon the presence of citrate, TPN^+ and GSSG, and the amount of GSSG reduced was the same in air or in N_2. When either Mg^{++} or AMP-5 were omitted from the reaction mixture (not shown), the amount of GSSG reduced was only slightly decreased. The final column of table II also shows the amount of O_2 taken up in the mixture exposed to air. Here again the reaction was dependent upon the presence of an oxidizable substrate. However, O_2 consumption was not dependent on added pyridine nucleotide to the same extent as was GSSG reduction for there was appreciable O_2 uptake in the absence of added pyridine nucleotides. The addition of TPN^+ and DPN^+ approximately doubled and tripled, respectively, the O_2 uptakes. The plant particles apparently contained enough pyridine nucleotides to support some hydrogen transfer to O_2, but these coenzymes were not available for hydrogen transfer to GSSG as the oxidant.

The effect of pH on O_2 uptake and on the coupled reduction of GSSG by the mitochondria preparations was investigated. With citrate as substrate, there was little reaction at pH 6.0 with either O_2 or GSSG as oxidant. Both reactions increased rapidly at pH 7.0 and then decreased again at pH 7.6. With malate as substrate, the reduction of GSSG again exhibited a sharp optimum at pH 7.0, but the reduction of O_2 remained independent of pH from 6.0 to 7.6. The observed optimum of pH 7.0 for the coupled reactions with O_2 or GSSG presumably reflects the overall effect of pH on the several enzyme systems involved, i.e., aconitase, isocitric dehydrogenase, cytochrome reductase, cytochrome c, cytochrome oxidase, and glutathione reductase.

The action of several metabolic inhibitors was also examined. As was expected, sodium azide and potassium cyanide, at a concentration of 0.001 M, inhibited the O_2 uptake 62 and 88 % respectively. However, azide and cyanide did not inhibit the reduction of GSSG, and diethyldithiocarbamate (0.001 M) did not affect either the O_2 uptake or GSSG reduction. Since azide and cyanide, but not diethyldithiocarbamate, are effective inhibitors of cytochrome oxidase (16), this enzyme appears to mediate the reduction of O_2 and it is reasonable to assume that a chain of cytochrome enzymes, similar to that found in animal mitochondria, exists in avocado particles. On the other hand, none of these inhibitors inhibit glutathione reductase (17), the enzyme responsible for linking the reduction of GSSG to the dehydrogenases in these preparations.

There is one interesting difference between the reduction of GSSG in air and the same reaction in N_2. The last line of table II demonstrates that DPN^+ will substitute in part for TPN^+ in the reduction of GSSG in air, but cannot substitute in N_2. In view of the fact that GSSG reductase of higher plants is TPN^+ specific (4), this observation can be explained if the DPN^+ is first phosphorylated to TPN^+ by ATP and if

TABLE II

REDUCTION OF OXIDIZED GLUTATHIONE BY CITRIC ACID

CONDITIONS	GSSG REDUCED MICRO-MOLES/MG N × HR		O_2 UPTAKE MICRO-ATOMS/MG N × HR
	N_2	AIR	
Complete system ..	2.55	2.56	4.5
Omit citrate	0.43	0.53	1.0
Omit TPN^+	0.29	0.18	2.2
Omit GSSG	...	0.03	4.1
Omit GSSG and TPN^+	0.04	0.01	2.7
Substitute DPN^+ for TPN^+	0.38	1.60	6.5

Complete system contained 0.02 M citrate; 1.5×10^{-3} M AMP-5; 10^{-3} M $MgSO_4$; 4.5×10^{-3} M GSSG; 6×10^{-5} M TPN^+; 0.01 M phosphate buffer, pH 7.1; 0.3 M sucrose; and 1 × washed avocado particles containing 1.04 mg N. Total volume 2.2 ml. Center well contained 0.1 ml 20 % KOH. Gas phase, air or N_2. Reaction time, 85 min.

the formation of ATP is dependent on aerobic conditions. Data supporting this explanation were obtained by comparing the amount of GSSG reduced in air and in N_2 when various combinations of pyridine nucleotides and adenosine phosphates were employed. Table III shows that DPN^+ together with AMP-5 partially substituted for TPN^+ and AMP-5 in air but not in N_2. When, however, ATP was supplied with DPN^+, reduction of GSSG occurred more readily both in air and in N_2. These results, in addition to indicating the phosphorylation of AMP-5 to ATP only under aerobic conditions, suggest the presence in avocado mitochondria of the enzyme described by Kornberg (18) which catalyzes the phosphorylation of DPN^+ to TPN^+ by ATP. The greater reduction of GSSG which occurred in N_2 as compared to O_2 in experiments 1 and 3 was regularly observed and may be due to the fact that, in the experiments carried out in N_2, there is only one oxidant available, namely GSSG.

THE OXIDATION OF GSH BY PLANT MITOCHONDRIA: In addition to catalyzing the reduction of GSSG in the presence of a suitable hydrogen donor, the mitochondria from avocado carried out the oxidation of GSH by O_2 under appropriate conditions. This oxidation is dependent upon the fact that avocado particles contained ascorbic acid oxidase which catalyzes reaction *2*. In a typical experiment (table IV), the

$$\text{Ascorbic acid} + \tfrac{1}{2}\,O_2 \rightarrow \text{Dehydroascorbic acid} + H_2O \qquad (2)$$

rapid and nearly complete oxidation of 11.4 micromoles of ascorbic acid was observed in 20 minutes in the presence of avocado particles. Appropriate blanks were obtained when the substrate was omitted and when the enzyme was heat inactivated. The latter experiment constitutes the control for any autoxidation of ascorbic acid which might occur due to the presence of metallic ions in the reaction mixture. The oxygen uptake was completely inhibited by 0.001 M diethyldithiocarbamate and demonstrates that the oxidation of ascorbic acid was mediated by a copper-containing enzyme, undoubtedly ascorbic acid oxidase.

TABLE III

SUBSTITUTION OF DPN^+ AND ATP FOR TPN^+ IN THE REDUCTION OF OXIDIZED GLUTATHIONE

EXPT NO.	COMPLETE SYSTEM WITH	GSSG REDUCED MICROMOLES/MG N × HR	
		AIR	N_2
1	TPN^+ and AMP-5	3.70	4.12
2	DPN^+ and AMP-5	1.22	0.61
3	DPN^+ and ATP	1.61	2.96
4	Omit TPN^+ or DPN^+	0.35	0.29

Complete system contained 0.02 M citrate; 1.5×10^{-3} M AMP-5 or ATP; 10^{-3} M $MgSO_4$; 3.3×10^{-3} M GSSG; 4.4×10^{-5} M TPN^+ or DPN^+; 0.008 M phosphate buffer, pH 7.1; 0.5 M sucrose; and 1 × washed avocado particles containing 0.83 mg N. Total volume, 3.0 ml. Center well contained 0.1 ml 20 % KOH. Gas phase, air or N_2. Reaction time 60 min.

TABLE IV

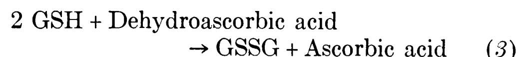
OXIDATION OF ASCORBIC ACID BY AVOCADO MITOCHONDRIA

CONDITIONS	O_2 UPTAKE µL IN 20 MIN
Complete system	123
Omit ascorbic acid	2
Heat inactivated	3
Diethyldithiocarbamate, 10^{-3} M	12

Complete system contained 3.8×10^{-3} M ascorbic acid; 7.5×10^{-3} M phosphate buffer, pH 7.0; 0.5 M sucrose; and 1 × washed particles containing 1.27 mg N. Total volume, 3.0 ml. Center well contained 0.1 ml 20 % KOH. Gas phase, air.

Cytochrome oxidase, which is also present in these particles, is inhibited only slightly by this concentration of diethyldithiocarbamate (16).

Although ascorbic acid oxidase will not oxidize GSH directly, dehydroascorbic acid, the oxidation product of ascorbic acid, will rapidly oxidize GSH at pH 7.0 and above according to reaction *3* (19).



$$2\ \text{GSH} + \text{Dehydroascorbic acid} \rightarrow \text{GSSG} + \text{Ascorbic acid} \qquad (3)$$

In addition, however, numerous investigators have shown that many plants contain an enzyme, dehydroascorbic acid reductase, which also catalyzes this reaction (see (2) for review). Since particles from avocado can catalyze the oxidation of ascorbic acid (reaction *2*) and since reaction *3* can occur both enzymatically and non-enzymatically, it was possible that the overall reaction, the oxidation of GSH by O_2 (reaction *4*) could occur in the presence of these par-

$$2\ \text{GSH} + \tfrac{1}{2}\,O_2 \rightarrow \text{GSSG} + H_2O \qquad (4)$$

ticles and catalytic amounts of ascorbic acid. Reaction *4*, together with the coupled reduction of GSSG already discussed, would then represent a path of hydrogen transport from substrate through pyridine nucleotides, GSH and ascorbic acid to O_2 which bypasses the cytochrome system.

Experiments testing the coupled oxidation of GSH with ascorbic acid and O_2 are presented in table V in which the results are expressed both as µl of O_2 taken up and micromoles GSH oxidized. In the presence of 1.1 micromoles of ascorbic acid, 17.7 micromoles of GSH were oxidized in 40 minutes. In the absence of ascorbic acid there was little reaction, and appropriate blanks (not shown) were obtained when the mitochondrial preparation was heat inactivated. The coupled oxidation of GSH was inhibited 65 % (based on O_2 uptake) by 0.002 M diethyldithiocarbamate due to the action of this inhibitor on ascorbic acid oxidase. On the other hand, 0.001 M iodoacetamide inhibited the coupled oxidation of GSH only 33 %. Since iodoacetamide is known to inhibit dehydroascorbic acid reductase (20), these data indicated that a substantial amount (67 %) of the reaction observed was probably due to the non-enzymatic oxidation of GSH by dehydroascorbic acid. Because of the rapidity of this

reaction at pH 7.0, it was impossible to obtain evidence for dehydroascorbic acid reductase in the avocado preparations.

The avocado particles were not unique in their ability to catalyze the reduction and oxidation of glutathione. Particles isolated from pea seedlings and from mung beans also carried out the reduction of GSSG (2). In addition, the coupled oxidation of GSH by ascorbic acid and O_2 was readily catalyzed by mitochondria obtained from pea hypocotyls.

EXPERIMENTS ON OXIDATIVE PHOSPHORYLATION: Biale and his associates (7, 14, 15) have demonstrated that avocado mitochondria will support phosphorylation during the oxidation of Krebs cycle intermediates. Since the work reported here has shown that GSSG can substitute for O_2 as an oxidant in these preparations, it seemed important to determine whether inorganic phosphate could also be esterified with GSSG as oxidant. Two different procedures for detecting phosphorylation were employed and numerous experiments were performed in which the experimental conditions were widely varied. However, all attempts to demonstrate oxidative phosphorylation with GSSG as the ultimate oxidant were unsuccessful. The initial experiments measured the disappearance of inorganic phosphate in reaction mixtures containing the necessary components for coupling the oxidation of citrate to the reduction of GSSG. Since the avocado particles contained hexokinase, it was only necessary to add glucose to provide a phosphate acceptor for any ATP which might be synthesized. Fluoride was included to prevent loss by phosphatase action. Duplicate vessels were run in N_2 and in air and the amount of inorganic phosphate esterified in each case was measured. While P : O ratios of approximately 1.0 were observed in air with citrate as the substrate, no significant uptake of inorganic phosphate occurred in N_2 with GSSG as the oxidant (table VI).

Although the oxidation-reduction potential of glutathione is not known with certainty, it is intermediate between that of diphosphopyridine nucleotide and that of cytochrome c (21). On thermodynamic grounds, therefore, the ratio of the number of atoms of phosphorus esterified to the number of moles of GSSG reduced (i.e., the P : GSSG ratio) could be 1.0. This figure is arrived at from a consideration of the oxidation-reduction potentials of the DPNH-DPN couple and the most negative value which appears possible for the GSH-GSSG couple (5). Since the P : O ratio observed in these experiments is only about 1.0, it was clear that a method of detecting phosphorylation was needed which was more sensitive than measuring the decrease in inorganic phosphate. In addition, greater sensitivity was also desirable in view of the fact that more citrate was oxidized with O_2 as oxidant than with GSSG. Accordingly, the method employed by Lehninger and his associates (11) in their early studies on oxidative phosphorylation was also employed. Results obtained by this method, which measures the amount of labeled inorganic phosphate incorporated into the esterified phosphorus fractions as a result of oxidative phosphorylation, are given for the experiment described in table VI. While 37 % of the P^{32} was esterified under aerobic conditions, only an insignificant amount of tracer was incorporated with GSSG as the sole oxidant. The failure to observe esterification could not be attributed to any inhibitory action of GSSG or GSH since these substances were added or were produced in the aerobic experiments.

Similar experiments with $P^{32}O_4^{\equiv}$ were carried out with mitochondria from avocado and peas in order to determine if phosphorylation occurred either during the oxidation of ascorbic acid or during the oxidation

TABLE V

OXIDATION OF REDUCED GLUTATHIONE BY AVOCADO MITOCHONDRIA

CONDITIONS	GSH OXIDIZED MICROMOLES	O_2 UPTAKE μL
GSH + ascorbic acid	17.7	119
GSH alone	0.6	11
GSH, ascorbic acid and 10^{-3} M diethyldithiocarbamate	6.2	43
GSH, ascorbic acid and 10^{-3} M iodoacetamide	14.4	82
Theory for GSH added	19.5	109
Theory for ascorbic acid added	1.1	13

Reaction mixture contained 6.5×10^{-3} M GSH; 4×10^{-4} M ascorbic acid; 0.5 M sucrose; 10^{-3} M $MgSO_4$; 7.5×10^{-3} M phosphate buffer, pH 7.1; and 1 × washed avocado particles containing 0.99 mg N. Total volume, 3.0 ml. Center well contained 0.1 ml 20 % KOH. Gas phase, air.

TABLE VI

OXIDATIVE PHOSPHORYLATION WITH AVOCADO MITOCHONDRIA

CONDITIONS	O_2 UPTAKE MICRO-ATOMS	INORGANIC P UPTAKE MICRO-ATOMS	P : O RATIO	GSSG REDUCED MICRO-MOLES	P^{32} ESTERIFIED %
Aerobic experiments					
1. Complete system	11.8	7.7	0.65	1.98	37.0
2. Omit citrate	1.9	0.9	...	0.51	1.3
3. Omit GSSG	12.1	11.6	0.96	0.14	37.0
Anaerobic experiments					
4. Complete system	...	1.8	...	1.54	1.9
5. Omit citrate	...	0.0	...	0.72	1.0
6. Omit GSSG	...	2.2	...	0.18	2.1

Complete system contained 0.02 M citrate; 1.5×10^{-3} M $MgSO_4$; 3×10^{-3} M AMP-5; 3.3×10^{-3} M GSSG; 4×10^{-5} M TPN^+; 0.01 M glucose; 0.01 M NaF; 0.007 M phosphate buffer, pH 7.4; 0.5 M sucrose; and 1 × washed avocado particles containing 1.02 mg N. Total volume, 3.0 ml. Center well contained 0.1 ml 20 % KOH. Gas phase, air or N_2. Each vessel received 7.7×10^5 cpm of $P^{32}O_4^{\equiv}$. Reaction time, 40 min.

of GSH in the presence of catalytic amounts of ascorbic acid. Again, however, negative results were obtained although control experiments with citrate as substrate and O_2 as oxidant demonstrated that the phosphorylation mechanisms in these preparations were intact and operative.

PARTICULATE GSSG REDUCTASE AND ASCORBIC ACID OXIDASE: The coupled reactions observed with plant mitochondria indicated the presence of GSSG reductase and ascorbic acid oxidase in these particles. While it is easy to demonstrate both enzymes qualitatively in avocado and pea mitochondria, attempts to determine quantitatively the amounts of GSSG reductase in these preparations have not been successful. The assay procedure used depended on coupling glucose-6-phosphate and glucose-6-phosphate dehydrogenase to the reduction of GSSG by the plant particles. Under conditions where reductase is limiting, the amount of GSSG reduced in a given time should be proportional to the amount of reductase employed (4). As shown in table VII, these conditions were not obtained with avocado mitochondria and similar effects were observed with particles from peas. The reaction was not proportional to the amount of particles added (expts. 1 and 2); moreover, when GSSG reductase from wheat germ (expt. 3) was added to the particles, the wheat germ activity was almost completely inhibited (expt. 4). Since the addition of AMP-5 greatly increased the amount of GSH formed (expt. 5) by avocado mitochondria, it seemed probable that the failure of the assay system to function properly was due to the splitting of TPN^+ by nucleotidases present in the mitochondria. Because of these difficulties, it has been possible to estimate only approximately the amounts of GSSG reductase present in the particle. Such approximate analyses indicate that the specific activity of well-washed avocado particles varied from one-fourth to more than one-half of the specific activity of the supernatant which contained the soluble proteins and submicroscopic fractions. Because of the inadequacy of the assay procedure, no attempt was made to conduct a careful study of the cellular distribution of GSSG reductase. However, it should be emphasized that this enzyme was not confined to the particulate fraction in the tissues studied, but was also found in the supernatant fraction containing the soluble proteins.

TABLE VII

ANALYSIS OF AVOCADO PARTICLES FOR GSSG REDUCTASE

EXPT NO.	ADDITIONS TO ASSAY MIXTURE	GSH FORMED MICROMOLES
1	0.2 ml Avocado mitochondria	2.85
2	0.4 ml Avocado mitochondria	2.76
3	0.9 mg Wheat germ GSSG reductase	8.10
4	(2) + (3)	2.97
5	As in (2) + 3×10^{-3} M AMP-5	7.74
6	As in (2), omit glucose-6-PO_4	0.15

Assay mixture contained 1.7×10^{-3} M glucose-6-PO_4; 3.3×10^{-3} M GSSG; 9×10^{-6} M TPN; 35 μgm/ml Zwischenferment; 0.05 M Tris buffer, pH 7.4. Avocado mitochondria (2.44 mg N/ml) were once washed. Total volume 3.0 ml. Time of incubation, 1 hr at 25° C.

In an effort to determine whether the GSSG reductase was firmly associated or attached to the plant particles, repeated washings with 0.5 M sucrose were carried out. These manipulations failed to dislodge the enzyme, and instead, soluble compounds containing nitrogen were removed thereby increasing the apparent specific activity of the particles with respect to GSSG reductase.

No attempt was made to determine the relative distribution of ascorbic acid oxidase in the plant tissues used in these experiments. There was abundant oxidase activity in the fraction representing the soluble proteins, and the amount of enzyme in the particles probably constituted only a small fraction of the total activity of the tissue. However, as in the case of GSSG reductase, the particulate ascorbic acid oxidase was not removed by extensive washing, and instead the specific activity appeared to increase with that treatment.

DISCUSSION

The reduction of GSSG catalyzed by plant mitochondria demonstrates that particulate dehydrogenases which oxidize Krebs cycle intermediates can be coupled with GSSG reductase. The dependency of the reduction of GSSG on TPN^+ is in keeping with the known specificity of GSSG reductase of higher plants for TPN^+ (4). No conclusions can be drawn, however, regarding the pyridine nucleotide specificity of the particulate dehydrogenases which coupled to the reduction of GSSG, since information is unavailable regarding the presence of the enzyme transhydrogenase (22) on plant mitochondria. The presence of transhydrogenase on the plant particles would permit hydrogen transfer to TPN^+ from DPNH formed by the action of DPN-specific dehydrogenases. It is also interesting that the oxidation of succinic acid could be coupled to GSSG reduction since there is no direct indication that pyridine nucleotides function in the oxidation of succinate. (There is evidence that DPNH can be oxidized by fumaric acid in the presence of a heart muscle preparation (32).) It is possible that the reduction of GSSG observed with succinate was due to the combined action of fumarase and malic dehydrogenase on the fumarate formed when succinate was oxidized. However, this is not in agreement with the observation that fumarate is only very slowly oxidized by the plant mitochondria.

The oxidation of GSH in the presence of plant particles and catalytic amounts of ascorbic acid demonstrates that these particulates possess a mechanism for the passage of hydrogen from substrate through pyridine nucleotides, glutathione and ascorbic acid to O_2. Mapson and Goddard (3) have previously shown that soluble extracts of pea meal can carry out the reduction of dehydroascorbic acid. Mapson and Moustafa (23) have recently extended these studies

and have observed the complete transfer system from substrate to O_2 in soluble extracts from pea seedlings. Their results differ from ours in one very important aspect: due to the presence of dehydroascorbic acid reductase in the soluble pea extracts, every step in their reaction sequence is enzyme catalyzed. Mapson and Moustafa have also concluded that the soluble GSSG-ascorbic acid pathway accounts for approximately 25 % of the total oxygen consumption of pea cotyledons.

Our results were unable to provide any evidence that the reduction and oxidation of glutathione by plant mitochondria is physiologically significant. The addition of catalytic amounts of GSH and ascorbic acid to mitochondrial preparations which were actively oxidizing succinic or citric acids by means of cytochrome oxidase did not result in an increased O_2 uptake. The rate of reduction of GSSG by plant particles was less than the rate of O_2 uptake observed in the same preparations. Finally, oxidative phosphorylation could not be observed either during the anaerobic reduction of GSSG or during the oxidation of GSH or ascorbic acid. Although these last observations indicate that particulate GSSG reductase and ascorbic acid oxidase cannot function in phosphorylation processes, the results obtained may have been due to the destruction of labile enzyme systems. These experiments on oxidative phosphorylation were performed with the hope of obtaining some additional information on the sites of phosphorylation in the hydrogen-transport scheme from pyridine nucleotide to O_2. Lehninger (24) has recently discussed this problem and work from his laboratory and from Lardy's (25) has provided considerable information.

The finding of GSSG reductase and ascorbic acid oxidase on plant mitochondria is worthy of comment. The former enzyme has been extracted as a soluble protein from wheat germ (4) and dried yeast (5) and is found in the soluble press juices obtained from fresh plant tissues (3, 6). Rall and Lehninger (5) concluded from tissue fractionation studies on rat liver that GSSG reductase was present mostly in the soluble fraction. In addition, Mapson and Moustafa (23) reported that the majority of GSSG reductase of pea seedlings was soluble. Nevertheless, the latter authors were able to observe the enzyme in mitochondria obtained from pea seedlings. The posibility of obtaining artifacts in the apparent localization of an enzyme within cells is well known (26). In plant tissues, these difficulties may be more pronounced due to the release of vacuolar contents during the homogenization of the tissue (27). Nevertheless, GSSG reductase was observed in significant quantities on mitochondria obtained from avocado fruit and pea seedlings. When these particles were isolated in a manner which maintained their structural integrity, extensive washing did not remove the enzyme but did solubilize other nitrogenous material in the particles.

It has recently been possible to isolate mitochondria from lupine cotyledons which carry out oxidative phosphorylation with P : O ratios approaching those observed in animal tissues (28). Such preparations presumably have maintained their structural integrity to a larger degree than the avocado particles which seldom exhibited P : O ratios above 1.0. However, the lupine mitochondria cannot link the reduction of GSSG to the oxidation of Krebs cycle acids even though the particles contain abundant amounts of GSSG reductase. These results therefore suggest that the hydrogen and electron transfer system can be shunted into GSSG reduction only in particles which have been altered considerably from their original state.

The cellular distribution of ascorbic acid oxidase has been discussed by Goddard and Stafford (29). Although usually considered as a soluble enzyme, there is recent evidence that ascorbic acid oxidase is also associated with cell wall material (30). Moreover, Mapson et al (31) have reported that mitochondria from pea seedlings will catalyze the oxidation of ascorbic acid.

Summary

1. The reduction of oxidized glutathione has been coupled to the oxidation of Krebs cycle intermediates in the presence of plant mitochondria.

2. The oxidation of reduced glutathione by molecular oxygen is catalyzed by plant mitochondria in the presence of catalytic amounts of ascorbic acid. This is due to the presence of ascorbic acid oxidase in the mitochondria.

3. With the demonstration of these reactions, a mechanism exists in plant mitochondria for the transfer of hydrogen from Krebs cycle substrate through pyridine nucleotides, glutathione, and ascorbic acid to O_2. Such a mechanism does not involve the participation of cytochromes.

4. Attempts to demonstrate oxidative phosphorylation during the reduction of oxidized glutathione and during the oxidation of reduced glutathione and ascorbic acid were negative.

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PINEAPPLE CHLOROSIS IN RELATION TO IRON AND NITROGEN[1,2]

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Pineapple plants grown in soils high in pH or in water-soluble manganese become chlorotic, because of greatly decreased iron availability. At high pH, the solubility of iron is greatly reduced and the physiological activity of iron is rendered ineffective by high concentrations of manganese (5, 25). In both cases, adequate supplies of iron, as solution sprays of ferrous sulfate, remedy the symptoms of chlorosis (30). Chlorosis also often results from inadequate supplies of nitrogen in the soil and may be remedied by additions of this element.

The paradox of the type of chlorosis that results

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