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Ascorbic Acid and Glutathione as Respiratory Carriers in the Respiration of Pea Seedlings
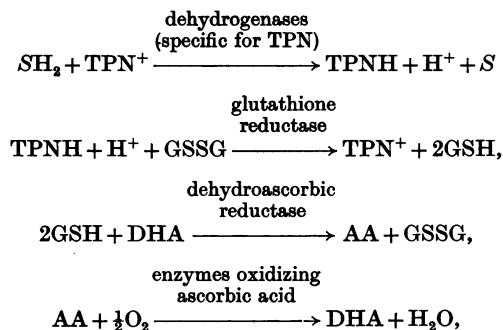
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Szent-Györgyi (1931), from a knowledge of the behaviour of reduced glutathione (GSH) and ascorbic acid (AA), first postulated a scheme whereby these substances might act as respiratory carriers. The subsequent discovery of the enzymes dehydroascorbic acid reductase (Crook & Hopkins, 1938; Crook, 1941) and glutathione reductase (Mapson & Goddard, 1951; Conn & Vennesland, 1951) made it possible to visualize a system whereby hydrogen may be transferred from substrates such as *iso*citrate and malate to molecular oxygen. Mapson & Goddard (1951), in fact, were able to demonstrate the reduction of dehydroascorbic acid (DHA) as a result of hydrogen transfer from either malate or *iso*citrate via triphosphopyridine nucleotide (TPN) and GSH. Such a system might be expected to consume oxygen if, in addition to these enzymes, a terminal oxidase capable of oxidizing

ascorbic acid was present. The reactions concerned may be formulated as follows:

$$SH_2 + TPN^+ \xrightarrow[\text{(specific for TPN)}]{\text{dehydrogenases}} TPNH + H^+ + S$$

$$TPNH + H^+ + GSSG \xrightarrow{\substack{\text{glutathione} \\ \text{reductase}}} TPN^+ + 2GSH,$$

$$2GSH + DHA \xrightarrow{\substack{\text{dehydroascorbic} \\ \text{reductase}}} AA + GSSG,$$

$$AA + \tfrac{1}{2}O_2 \xrightarrow{\substack{\text{enzymes oxidizing} \\ \text{ascorbic acid}}} DHA + H_2O,$$

where SH_2 represents an oxidizable substrate.

The work described here aimed to demonstrate the presence of such a system in extracts *in vitro*, and to evaluate its importance in the respiration of the tissue *in vivo*.

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EXPERIMENTAL

Preparation of materials and reagents

Pea seedlings. Pea seeds, var. Kelvedon Wonder, were sterilized by soaking in 1% (w/v) $HgCl_2$ solution for 1 min., washed thoroughly in running tap water, soaked for 24 hr. in tap water and then germinated for the appropriate period at 20°. In the designation of age of seedling the soaking period is included as the first day of germination.

Triphosphopyridine nucleotide (TPN). The method of preparation of Warburg, Christian & Griese (1935), as modified by Haas (unpublished), was normally used. In some cases the method of LePage & Mueller (1949) was employed. The purity was determined according to the method used by LePage & Mueller (1949), except that the enzyme *isocitric dehydrogenase* was used instead of glucose 6-phosphate dehydrogenase.

Dehydroascorbic acid (DHA). This was prepared by dissolving the equivalent amount of ascorbic acid in distilled water and adding bromine drop by drop until a faint orange colour of bromine appeared. The excess bromine was removed by aeration and the solution was brought to pH 6·5 by the addition of a solution of 20% (w/v) Na_2HPO_4.

Glutathione. The reduced form (GSH) was obtained from British Drug Houses Ltd. Oxidized glutathione (GSSG) was prepared by dissolving the appropriate amount of the reduced form in water, adding a drop of starch indicator and titrating with an iodine solution until a very faint blue colour appeared. This solution was freshly prepared before each experiment.

Sodium isocitrate. This was prepared from DL-*isocitric* acid lactone, obtained from H.M. Chemical Co. Ltd., California, U.S.A. The lactone solution was heated at 100° with the equivalent amount of NaOH for 20 min. This solution was stored at −20°.

Sodium malate. This was prepared from malic acid, purchased from Hopkin & Williams Ltd.

Methods

Oxygen uptake. Oxygen consumption was measured in Warburg manometers at 25° in air. The fluid vol. was 3 ml., including 0·2 ml. of 20% KOH in the centre well to absorb CO_2. The flasks were equilibrated by shaking for at least 10 min. The reaction was started by tipping the substrate from the side arm.

Fractionation of cell constituents. As the mitochondria are well known to be sensitive to high temperature, all apparatus and solutions were cooled to 1° before use, and the extracts obtained kept at this temperature until the start of the experiments. Germinated pea seeds (60 g.) were ground in a mortar to a fine paste with 80 ml. of 0·1 M phosphate buffer, pH 7·4, containing 0·4 M sucrose and 0·004 M-$MgSO_4$. The extract was then centrifuged at 500 g for 5 min. The residue, which contained most of the starch and cell debris, was discarded and the supernatant, referred to in the text as the 'suspension', was recentrifuged at 20 000 g for 20 min. at −3°. Under these conditions, the cytoplasmic particles (mitochondria) are sedimental, and the supernatant represents the soluble part of the cytoplasm. The mitochondria were washed by suspending in 60 ml. of the same sucrose buffer solution and recentrifuged at 20 000 g for 20 min. They were then taken up in a volume of sucrose buffer equal to that of the original extract.

The respiration of these cell fractions was determined as quickly as possible after their preparation, usually within 1 hr. of starting extraction, and the same applied to the extracts used for studying the rate of reduction of DHA.

Determination of ascorbic acid. Direct visual titration against 2:6-dichlorophenolindophenol was used according to the method of Harris & Olliver (1942).

Determination of reduced glutathione. GSH was determined by titration against potassium acid iodate, using the method of Fujita & Numata (1938).

Determination of enzyme activity. Ascorbic oxidase activity was measured by determining the catalytic effect of the extract on the oxidation of ascorbic acid at 25°. The rate of oxidation of the substrate was followed by removal of samples into metaphosphoric acid, and determining the unoxidized ascorbic acid by titration against indophenol dye.

The estimation of the activity of dehydroascorbic acid reductase was carried out by determining the rate of formation of ascorbic acid on adding GSH (3 mg./ml.) and DHA (1 mg./ml.) to the extract under anaerobic conditions.

Glutathione reductase activity was measured by determining the rate of formation of GSH on the addition of malate, TPN, $MnCl_2$ and GSSG, under anaerobic conditions according to the method described by Mapson (1953).

Determination of CO_2 production by intact seeds. The CO_2 produced by 10–15 g. of seedlings was absorbed in 0·2 N-$Ba(OH)_2$ solution, the CO_2 being determined by back titration with standard HCl.

RESULTS

If ascorbic acid and glutathione act as respiratory carriers in a scheme similar to that suggested in the introduction, it should be possible to demonstrate the consumption of oxygen by extracts in which all the enzymes of this system are present. One tissue in which all these enzymes could be demonstrated was pea seeds which had been allowed to germinate for 5 days at 20° and which were extracted as follows: Pea seedlings (50 g.) were ground with 50 ml. of 0·1 M phosphate buffer, pH 6·8, and left at room temperature for 15 min. with occasional stirring. The mixture was then centrifuged at 500 g for 5 min., and the supernatant was recentrifuged at 20 000 g for 30 min. The clear final supernatant was dialysed at 1° for 72 hr. against 5 l. of 0·1 M phosphate buffer, pH 6·8, which was changed every 24 hr. It was necessary to prolong the dialysis for this period in order to remove completely substrates and/or coenzymes to reduce oxygen uptake to zero. Air was bubbled through the outside solution to facilitate dialysis.

In this enzyme preparation the presence of malic enzyme, GSSG reductase, DHA reductase together with ascorbic acid oxidase was established in separate experiments. Further, DHA, when added to the preparation, was reduced under anaerobic conditions.

As shown in Fig. 1, the enzyme preparation without the addition of any substrate or coenzyme showed no oxygen uptake. Five different experiments were carried out in order to reveal the presence of a GSH–AA hydrogen-transfer system. The substrates and coenzymes added to the enzyme extract which contained 4×10^{-3}M-Mn^{2+} or -Mg^{2+} were as follows: (1) AA and GSH; (2) malate and TPN; (3) malate, TPN and AA; (4) malate, TPN and GSH; and (5) malate, TPN, GSH and AA.

of the system were present, was there a rapid and continuous oxygen uptake. After 3 hr. the experiments were discontinued and the contents of the Warburg flasks analysed for ascorbic acid; only in the flask containing the complete system was any ascorbic acid detected.

Essentially similar results were obtained when DHA and GSSG were substituted for ascorbic acid and GSH, save that the small initial oxygen uptake due to the oxidation of the reduced forms was eliminated.
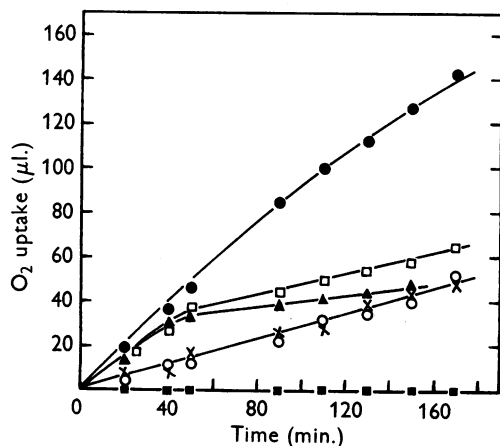

Fig. 1. Oxygen uptake due to a GSH–ascorbic acid enzyme system in dialysed extracts from 5-day germinated peas. Each flask contained 2 ml. of enzyme extract in a volume of 2·8 ml. ■, Dialysed extract; ○, extract + malate (2 mg./ml.) + TPN (30 μg./ml.); ×, extract + malate + TPN + GSH (50 μg./ml.); □, extract + malate + TPN + AA (50 μg./ml.); ▲, extract + GSH + AA; ●, extract + malate + TPN + AA + GSH.
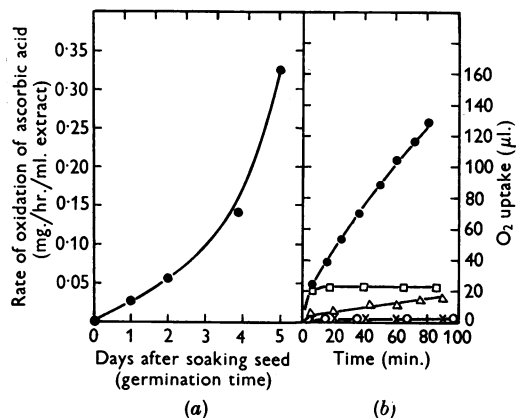
Fig. 2. Development of ascorbic acid oxidase activity during germination, and the effect of adding this enzyme on the oxygen consumption of extracts prepared from pea seeds in the early stages of germination. Concentrations of TPN, malate, GSH and ascorbic acid are as in Fig. 1. (a) Ascorbic acid oxidase activity of pea-seed extracts at different stages of germination. (b) O$_2$ uptake of dialysed extracts prepared from pea seeds after soaking for 24 hr. ×, Dialysed extract; ○, extract + AA oxidase preparation; □, extract + AA oxidase preparation + AA + GSH; △, extract + TPN + malate + GSH + AA; ●, extract + AA oxidase + TPN + malate + GSH + AA.

Expt. 1 showed that on the addition of ascorbic acid and GSH an initially rapid but brief oxygen uptake was observed, which was equivalent to the amount required to oxidize both the ascorbic acid and GSH added. It appears, therefore, that there was no regeneration of ascorbic acid or GSH under these conditions.

In Expt. 2, on the addition to the enzyme preparation of malate and TPN, a low but continuous oxygen uptake was observed which may have been due to hydrogen transfer from TPNH via flavoprotein enzymes to molecular oxygen, for it was not abolished by 10^{-3}M cyanide. In Expt. 3, on the further addition of ascorbic acid, oxygen consumption was increased initially only by the amount required to oxidize the ascorbic acid. In Expt. 4, when GSH was added the rate of oxygen uptake was no greater than that observed in Expt. 2, indicating that in the absence of ascorbic acid or its oxidized product no oxidation of GSH occurred. Only in Expt. 5, where all components

Attempts to demonstrate the presence of such a system in dialysed extracts obtained from pea seeds in the very early stages of germination (soaked for 1 day) were unsuccessful. This was apparently due to the absence of an enzyme catalysing the oxidation of ascorbic acid, for the rate of oxidation of this substance when added to extracts prepared from such seeds was low. This is shown in Fig. 2 (a), where the development of ascorbic acid oxidase as the seed germinates is illustrated. The assumption that the failure to demonstrate the GSH–ascorbic acid system was due to lack of a suitable terminal oxidase was found to be correct by showing (1) that all the other enzymes of the system were present, (2) that on addition of DHA, GSSG, TPN and malate, ascorbic acid was formed, and (3) that the addition of an

ascorbic acid oxidase preparation from cucumber (Crook, 1941) led to an immediate consumption of oxygen, which, as before, was shown to be due to the participation of GSH and ascorbic acid (Fig. 2b).

The consumption of oxygen, due to the operation of the GSH–ascorbic acid system, was also shown to occur in dialysed extracts prepared from peas germinated for longer periods (6–7 days). It was noteworthy, however, that as the germination of the seedlings progressed, the oxygen uptake stimulated by the addition of TPN and malate alone to the enzyme extract increased relatively to the oxygen uptake directly attributable to the GSH–ascorbic acid system. Results showing this are given in Table 1. It seems clear that as germination progresses the activity of enzymes capable of catalysing the direct oxidation of TPNH (for convenience collectively termed 'diaphorases') increases relatively to the activity of the enzymes associated with the reduction and oxidation of GSH and ascorbic acid.

Table 1. *Proportion of oxygen uptake of dialysed extract of peas attributable to 'diaphorase' enzymes and enzymes associated with GSH–ascorbic acid system*

Dialysed extract prepared from	Percentage of oxygen uptake due to	
	Diaphorase system	GSH–ascorbic acid system
2-Day germinated peas + ascorbic oxidase	20	80
5-Day germinated peas	40	60
7-Day germinated peas	58	42

The fact that these enzymic reactions can be demonstrated in plant extracts *in vitro*, however, gives no indication to what extent the normal respiration of the tissue proceeds by this route *in vivo*.

Intracellular localization of enzymes oxidizing and reducing glutathione and ascorbic acid in pea seedlings

It was thought that a knowledge of the localization of the enzymes associated with the GSH–ascorbic acid system within the cell might be of interest in evaluating the importance of the system in respiratory processes. The activity of each of the enzymes specifically associated with this system (i.e. ascorbic acid oxidase, dehydroascorbic acid reductase and glutathione reductase) was accordingly determined in (a) the suspension (mitochondria + soluble part of the cytoplasm), (b) the mitochondria, and (c) the soluble part of the cyto-

plasm as obtained from pea seeds which had been germinating for 1, 5 or 7 days. The presence of all these enzymes could be demonstrated in the suspension prepared from such seeds. The activity in the suspension of dehydroascorbic acid reductase could be entirely attributed to its activity in the soluble part of the cytoplasm, which was confirmed by the observation that intact mitochondria prepared from seedlings after 1, 5 or 7 days germination failed to catalyse the reduction of DHA. Enzymes catalysing the reduction of GSSG or the oxidation of ascorbic acid were also mainly concentrated in the soluble part of the cytoplasm. An oxidase catalysing this oxidation, although absent from the mitochondria prepared from seedlings after 1-day germination, was present in these particles after 7 days germination. However, even in the latter case, it accounted for only 10% of the total oxidase activity of the suspension.

Our failure to demonstrate any appreciable ability of mitochondria to reduce DHA and GSSG may have been due to either the inaccessibility of added substrates to the enzymes present in the intact mitochondria, or to the absence of the enzymes from these cytoplasmic particles. It was essential, for our purpose, to decide which of these interpretations was correct. Accordingly, we attempted to disintegrate the mitochondria by methods which have been used by many workers to prepare soluble enzymes from cytoplasmic particles. Three methods were used: (1) treatment with cold acetone at −20° (Hogeboom, Claude & Hotchkiss, 1946; Drysdale & Lardy, 1953), (2) addition of surface-active agents such as octan-2-ol (Huennekens & Green, 1950), and (3) homogenization in a Waring Blendor (Still & Kaplan, 1950).

The treatment with acetone was carried out as follows: Mitochondria were washed with sucrose buffer and suspended in a small volume of sucrose–phosphate buffer. Excess of cold acetone at −20° was then added and the mixture was centrifuged. The acetone layer was decanted and a stream of nitrogen was passed into the centrifuge tube to remove the remaining acetone. The residue was then taken up in the appropriate volume of sucrose buffer and used for enzymic tests.

In the second method, 0·02 ml. of octan-2-ol was usually added to each ml. of mitochondrial preparation.

In the third method, homogenization was carried out in a micro-Waring Blendor for 6 min. at 1–5°.

It was first necessary to show that the enzymes themselves were not affected by any of these treatments. The enzymes in the soluble fraction of the cytoplasm were, therefore, subjected to each of these procedures and the activity was tested before

and after. The results showed that none of these treatments had any adverse effects on ascorbic acid oxidase or dehydroascorbic acid reductase. Gluta-thione reductase was not affected by either the octan-2-ol or acetone treatment, but there was a slight decrease (30%) in its activity after blending.

No evidence for the presence of an enzyme cata-lysing the reduction of DHA was obtained when mitochondria were subjected to these treatments, nor was the oxidase activity associated with intact mitochondria increased by any of them. It there-fore seemed that the enzyme specifically involved in the reduction of DHA was associated with the soluble part of the cytoplasm and not with the mitochondria.

In contrast to the conclusions of Rall & Lehninger (1952) for liver and of Davies (1954) for peas, we have found glutathione reductase in pea mitochondria, but its activity was at the most only about one-fifth of its activity in the soluble fraction.

Relation between the oxygen consumption of pea suspensions and the respiration of intact pea seedlings

The fact that the enzymes associated with the reduction of DHA and GSSG are mainly located in the soluble part of the cytoplasm made it possible to determine more closely the part played by the system in total respiration. Thus electron transfer via the system could not be more than the total respiratory activity of the soluble part of the cyto-plasm itself. If, therefore, the respiratory activities of this fraction and of the mitochondria could be separately estimated, the role played by the GSH–ascorbic acid system could be more closely defined.

It was first necessary to determine whether during extraction of the different cellular fractions the respiration of the tissue had been seriously affected, because unless the respiration of the freshly prepared suspension is of the same order as that of the intact tissue, the results may obviously rest on an erroneous basis. Secondly, the pro-

cedure used in the further separation of mito-chondria and soluble fraction must be such that the sum of the respiration of the separated fractions is equal to that of the suspension. The following experiments were accordingly carried out to establish these points.

Pea seeds were germinated at 20°. After 5 days, 10–15 g. of seedlings were incubated at 25° and their CO_2 production was measured. At the same time a further sample was extracted as described earlier, and the oxygen uptake of the suspension determined in the Warburg apparatus. On the assumption that the R.Q. was 1·0, the respiration of the intact seedlings was expressed as μl. of oxygen/ g. fresh weight of seedlings/hr., and the oxygen uptake of the suspension similarly. The results showed that the respiration of the suspension was of the order of 83% of that of the intact tissue. There are reasons, however, for believing that this estimate is low. In the preparation of suspensions from the seedlings, large amounts of starch and cell debris are discarded. Some of the mitochondria are, no doubt, lost with the cell debris or disinte-grated during the preparatory procedures. There is no direct method of determining this loss, which Millerd (1953) thought might be as high as 50%. Some further experiments were, therefore, carried out in an attempt to allow for this. In these experiments the nitrogen content, as representing the total protein content, was taken as the basis for the calculation of the oxygen consumption. The results obtained (Table 2) show an even closer agreement between the oxygen consumption of the suspension and that calculated for the intact tissue.

In the preparation of these tissue suspensions, besides disruption of the cells some dilution of the constituents of the protoplasm was inevitable. We have tried to find out whether this dilution had affected the oxygen uptake. It was shown that a further twofold dilution of suspensions, prepared either from cotyledons or detached embryos, reduced the oxygen consumption strictly in accordance with the degree of dilution. There is

Table 2. *Comparison of the oxygen consumption of whole seedlings, cotyledons and detached embryos, with that of suspensions prepared from these tissues*

Material	Intact tissue		Suspension O_2 uptake equivalent to 1 g. of tissue (μl./g./hr.)	Intact tissue		Suspension O_2 uptake equivalent to 1 g. of tissue (μl./mg. N/hr.)
	O_2 uptake calc. from CO_2 production (μl./g./hr.)	O_2 uptake measured directly (μl./g./hr.)		O_2 uptake calc. from CO_2 output (μl./mg. N/hr.)	O_2 uptake measured directly (μl./mg. N/hr.)	
Whole seedling (5 days)	242*	—	210	13·4	—	13·1
Cotyledon (5 days)	250*	321, 252	220	12·7	16·3 12·7	12·1
Detached embryo (5 days)	664†	695, 695	83	96·5	101, 101	15·3
Cotyledon (7 days)	—	300, 246	200	—	19·4, 15·8	13·8
Detached embryo (7 days)	—	684, 684	84	—	107, 107	17·9

* Mean of hourly readings over a 6 hr. period. † Mean of hourly readings over a 2 hr. period.

thus no evidence that the oxygen consumption of these suspensions was altered in other respects merely as a result of any dilution of the cell cytoplasm.

Cotyledons were detached from the rest of the embryo (plumule and radicle) and the experiments were repeated with both these tissues. In addition, the oxygen uptakes of the intact cotyledon and of the detached embryo were directly determined in the Warburg apparatus; there was good agreement, considering the size of samples used (1 g.), between the oxygen uptake thus measured, and the oxygen consumption as calculated from the CO_2 production of 10–15 g. of these tissues. A comparison of the respiration of the intact tissues with the oxygen consumption of the corresponding suspensions revealed an important difference between the cotyledon and detached embryo. With the former, the oxygen consumption of the intact tissue, whether calculated from the CO_2 production or by direct measurement, agreed reasonably well with that of the suspension, but with the latter the oxygen consumption of the suspension was only about one-tenth of that of the intact tissue (Table 2). Clearly, in the latter case the oxygen consumption had been altered so much during extraction that we felt it impossible from results with such extracts to draw any conclusions about the part played by the GSH–ascorbic acid enzyme system in the respiratory activity. Nevertheless, as shown later, we have analysed the respiration of suspensions from the detached embryo in the same way as that used for similar extracts from the cotyledon, in the hope that the results might be of some interest. The good agreement reported above for the oxygen consumption of the intact whole seedling with that of suspensions was obviously due to the fact that the cotyledon comprised 87 % of the weight of the seedling at this stage of germination (5 days).

Oxygen consumption of the mitochondria and soluble fraction of the cytoplasm

Having established for the cotyledon that the procedure used to extract these cellular components did not appear to alter significantly their respiratory activity, we proceeded to separate mitochondria from the soluble fraction of the suspension and to determine their separate oxygen consumptions. When this was carried out on peas germinated for 5 days it was found that the sum of the oxygen consumption of washed mitochondria and of the soluble fraction amounted to only 70 % of the total oxygen consumption of the suspension. This discrepancy appears to be due to an artificial depression of the respiration of the mitochondria due to a lack of substrates and/or coenzymes. Millerd (1953) determined the oxygen uptake of

washed mitochondria after adding succinate in arbitrary amounts, and this was taken to indicate the respiration of these cytoplasmic particles under physiological conditions. A better evaluation of the respiration of mitochondria under physiological conditions would appear to be obtained if the substrates, as distinct from the enzymes, in the soluble part of the cytoplasm were added to these cytoplasmic particles. In this case we should be determining the oxygen uptake in the presence of the substrates which are normally in contact with the mitochondria in the living cell. This was achieved by measuring the oxygen uptake of washed mitochondria in the presence of the soluble part of the cytoplasm, in which the enzymes had previously been inactivated by heating for 2 min. at 100°. When this was done the oxygen consumption of the mitochondria was increased to a value which was 2–3 times that of the washed mitochondria alone (Fig. 3).
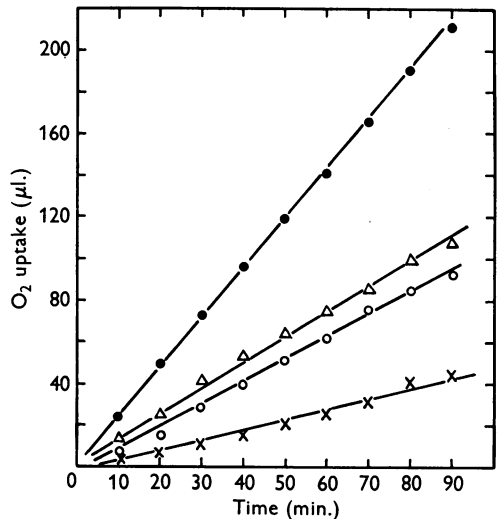

Fig. 3. Oxygen uptake of cellular fractions from 5-day germinated seeds. ●, Mitochondria + soluble part of cytoplasm (suspension); ○, mitochondria + heated soluble part of cytoplasm; ×, washed mitochondria in equivalent concentration; △, equivalent amount of soluble part of cytoplasm.

This technique was applied to pea seeds which had been germinated for 2, 5 and 7 days and the oxygen consumption of the suspension, mitochondria and soluble fraction determined. The oxygen uptake of the two latter fractions on summation accounted for 90, 97 and 99 % respectively of the total respiration of the suspension prepared from the same seedlings.

This method of estimating the oxygen consumption of the mitochondria and of the soluble

fraction was further checked by determining the oxygen consumption of the suspension and soluble fractions directly and obtaining that of the mitochondria by difference; results by either method agreed extremely well.

The distribution of the oxygen uptake between these cell fractions prepared from the cotyledon was found to be almost equally divided between the mitochondria and soluble fraction. It follows from this, and from the distribution of the enzymes associated with the GSH–ascorbic acid system, that the latter cannot account for, at the most, more than half of the respiration of the cotyledons.

In similar experiments with suspensions from detached embryos the oxygen consumption of the soluble fraction remained fairly constant over the period studied (5–9 days), but that of the mitochondria progressively increased. At 5 days 70% of the total respiration of the extract resided in the soluble fraction, but this decreased to a value of 50% over the next 4 days.

Inhibitor studies

Having shown that the participation of the GSH–ascorbic acid system in the transfer of electrons cannot be greater than that represented by the oxygen uptake of the soluble part of the cytoplasm, we used enzyme inhibitors in an attempt to determine the proportion of the oxygen consumption of this cell fraction which could properly be ascribed to this enzyme system. Since we found in our extracts no enzymes capable of oxidizing GSH in the absence of dehydroascorbic acid, the whole of the electron transfer must proceed via ascorbic acid. It followed that the easiest way of blocking the system was to prevent the functioning of those enzymes which oxidize ascorbic acid. In peas the oxidases most likely to be involved are ascorbic oxidase and cytochrome oxidase. Fortunately the latter enzyme appears to be exclusively confined to the mitochondria; certainly, we have found no evidence for its presence in the soluble part of the cytoplasm. We could therefore employ reagents such as cyanide or sodium diethyldithiocarbamate as inhibitors, without fear that the inhibition observed was due to interference with other respiratory pathways involving cytochrome oxidase.

If it could be shown that the maximum degree to which the oxygen consumption of the soluble fraction is depressed is the same for different inhibitors all capable of inactivating copper proteinates we should have suggestive evidence that the inhibition observed was due to the inactivation of enzymes capable of oxidizing ascorbic acid, and therefore that the fall in oxygen uptake was due to inactivation of an ascorbic acid system.

It was first essential to determine which of the known inhibitors of copper proteinates would prevent the oxidation of ascorbic acid in our extracts. Of those tested cyanide, diethyldithiocarbamate and 8-hydroxyquinoline all inhibited at millimolar concentration in extracts prepared from seedlings at all stages of germination studied. The inhibition with cyanide and diethyldithiocarbamate was complete at this concentration but with 8-hydroxyquinoline the percentage inhibition was somewhat less (88%).

The oxygen consumption of the soluble fraction of the cytoplasm prepared from pea seedlings after 2, 5 and 7 days germination was accordingly determined in the presence of cyanide and of diethyldithiocarbamate. In all cases the maximum percentage inhibition observed with both compounds was, within experimental error, the same and was obtained at concentrations above 5×10^{-4}M (Table 3a).

The fact that the oxygen consumption of all these extracts was affected to a similar degree by cyanide and diethyldithiocarbamate makes it probable that much of the respiration is normally proceeding through oxidases containing copper as the prosthetic group, and of these the most likely to be involved are those concerned with the oxidation of ascorbic acid.

Reduction of DHA by soluble fraction of cytoplasm

Although such results are most simply explained on the assumption that cyanide and diethyldithiocarbamate have prevented the functioning of a respiratory chain involving ascorbic acid, it cannot be accepted, on this evidence alone, that the GSH–ascorbic acid system is responsible for the whole of the cyanide-sensitive respiration of the soluble part of the cytoplasm.

The work of Matthews (1951), Nason, Wosilait & Terrell (1954) and Kern & Racker (1954) has shown the existence of an enzyme in peas catalysing electron transfer from DPNH to an acceptor which appears to be an intermediate oxidation product between ascorbic and dehydroascorbic acid; in these reactions dehydroascorbic acid cannot act as an acceptor. It is thus possible that some of the cyanide-sensitive respiration of the soluble fraction is due to this system, since, like the GSH–ascorbic acid system it would not function in the absence of enzymes oxidizing ascorbic acid.

In an attempt to obtain further evidence, we have estimated the rate of hydrogen transfer through the GSH–ascorbic acid system by measuring the rate of reduction of DHA when this substance was added to the soluble enzyme fraction. This may be most simply accomplished by measuring the rate of reduction in the absence of oxygen. Such a procedure, while preventing the

oxidation of ascorbic acid by ascorbic acid oxidase, also measures the full reducing potential of the extract towards DHA, but at the same time may give erroneous results since the action of diaphorase enzymes oxidizing TPNH and therefore normally acting competitively with glutathione reductase would be eliminated by exclusion of oxygen. A better, though by no means ideal, evaluation of the rate of reduction can, however, be obtained by using cyanide under aerobic conditions, in which circumstances oxidation of ascorbic acid is prevented, without the total exclusion of other enzyme systems. The presence in wheat of TPNH-oxidizing enzymes which are partially inhibited by 10^{-3}M cyanide has been shown by Conn, Kraemer, Liu & Vennesland (1952) and by Humphreys (1955).

Analysis of the soluble fraction of the cytoplasm prepared from the whole seedling

We have used these techniques in an attempt to evaluate the importance of the GSH–ascorbic acid system in the respiration of the developing seedling. In the first instance this analytical approach was applied to the soluble fraction of the cytoplasm obtained from the whole embryo (cotyledon, plumule and radicle) with results (Table 3b) which showed that the oxygen uptake as computed from the rate of reduction of DHA, accounted for the major proportion (70%) of the respiration inhibited by cyanide and diethyldithiocarbamate. In the earlier stages of germination the rate of reduction of DHA was identical under anaerobic or aerobic conditions where cyanide was present to

Table 3. *Effect of 10^{-3}M cyanide and diethyldithiocarbamate on the respiration of the soluble fraction of the cytoplasm prepared from suspensions of whole seedlings and the rate of reduction of DHA by the same fraction*

(a)

O₂ uptake expressed — O$_2$ uptake of soluble part of cytoplasm (μl./g. fresh wt./hr.)

Material	Total	Cyanide-insensitive	Cyanide-sensitive	Inhibition of respiration by cyanide (%)	Diethyldithio-carbamate-insensitive	Diethyldithio-carbamate-sensitive	Inhibition of respiration due to diethyldi-thiocarbamate (%)
2-Day seedling	10	0	8	80	0	8	80
2-Day seedling (with added AA oxidase)	53	4	49	92	6	47	89
5-Day seedling	78	24	54	69	26	52	67
7-Day seedling	95	56	39	41	60	35	37

(b)

	Rate of reduction of DHA by the soluble part of cytoplasm expressed as equivalent of μl. O$_2$/g. fresh wt./hr.			Respiration as measured by rate of reduction of DHA in air with KCN	
Material	In N$_2$	In air with 10^{-3}M-KCN	Rate in air as % of that in N$_2$	Percentage of cyanide-sensitive respiration of soluble fraction	Percentage of total respiration of soluble fraction
2-Day seedling	34	34	100	79*	64*
5-Day seedling	39	37	96	69	47
7-Day seedling	40	28	70	72	30

* Calculated values obtained from reduction of DHA by extract, with respiration of extract with added ascorbic acid oxidase.

It was further of importance to ascertain that the rate of reduction was independent of the concentration of DHA over the range tested (0·03–0·40 mg./ml. reaction mixture), which suggests that in these extracts the rate-limiting step in the transfer of hydrogen through the GSH–ascorbic acid system is the rate of reduction of GSSG rather than that of DHA.

prevent oxidation of the ascorbic acid formed; only with older seedlings was there any marked influence of oxygen on this reaction. The results included in Table 3b show that the rate of reduction of DHA with 2-day germinated peas was as great as that observed with older peas, suggesting that the low oxygen consumption of the soluble fraction prepared from the former peas was not

due to any deficiency of substrates, enzymes or coenzymes associated with the reduction of GSSG or DHA but rather to the lack of enzymes capable of oxidizing ascorbic acid. This was subsequently confirmed by the observation that the respiration could be greatly increased by the addition of ascorbic acid oxidase (Table 3a).

Activity of the GSH–ascorbic acid system in cotyledons and detached embryos

The above results gave only a very approximate overall picture of the role played by this enzyme system in the developing seedling, and no attempt was made to determine whether the system was operating in the respiration of the cotyledon as distinct from the detached embryo (plumule + radicle detached from the cotyledon). Unless the enzymes associated with oxidation and reduction of GSH and ascorbic acid, and other enzymes which may act competitively with this system, are equally distributed throughout these tissues, the results obtained from an extract of the mixed tissue may be misleading.

One difference between the cotyledon and the detached embryo was the very uneven distribution of ascorbic acid oxidase. We have already shown the development of this enzyme in extracts prepared from whole seedlings, and we were therefore surprised to find that although this enzyme did increase in the cotyledon during germination its activity in this tissue was only about 5% of its activity in extracts from the detached embryo. Whilst the activity of the oxidase in the soluble fraction of the cytoplasm prepared from cotyledons was only just adequate to account for the cyanide-sensitive respiration of this fraction, its activity in similar extracts from the detached embryo was some fifty times greater than that necessary to account for the cyanide-sensitive respiration observed. Assuming the oxygen consumption of these extracts, though reduced in magnitude, to be representative of the oxygen consumption of the intact tissue, the cyanide-sensitive component would be about ten times that observed, in which case the activity of the oxidase would be only five times that necessary for an enzyme acting as a terminal oxidase. Moreover, it has to be remembered that the assay of the ascorbic acid oxidase activity is necessarily carried out on dialysed extracts, in order to eliminate the reducing systems; hence the observed enzyme activities, especially the very high activity of extracts from detached embryo, may be very different from their real activity in vivo. It would seem worth while to pursue this point further in subsequent work.

We found that the other enzymes associated with the GSH–ascorbic acid system were present in extracts of the soluble fraction of the cytoplasm prepared from either cotyledons or detached embryos, as shown by the ability of such extracts to reduce DHA under anaerobic conditions. Within a period of 5 days after the start of germination there was no difference in the rate at which DHA was reduced between comparable extracts prepared from either the cotyledons or detached embryos but, whilst the activity of the former remained constant throughout the period of germination studied (9 days) the activity of the detached embryo tissue decreased by about half in the later stages (Table 4, col. 6). A further difference was that, whilst oxygen did not affect the rate of reduction of DHA in extracts from cotyledons containing cyanide, it reduced the rate of the reaction in extracts from the detached embryo, indicating that the cyanide-insensitive diaphorase enzymes oxidizing TPNH, already referred to, were absent from the cotyledons but present in the detached embryo. It was therefore to be expected that cyanide would inhibit the oxygen uptake of the soluble fraction of the cytoplasm from the cotyledons to a greater extent than from detached embryos, and this was found to be so (Table 4, col. 5). With 5- to 9-day-old cotyledons 70% of the total oxygen uptake of the soluble fraction was cyanide-sensitive and, from the rate of reduction of DHA in air with cyanide (Table 4. col. 7), 60% of this could be ascribed to electron transfer via GSH and ascorbic acid (Table 4, col. 9), which means that a little less than half of the total oxygen consumption of the soluble fraction of the cytoplasm may pass over this route (Table 4, col. 10).

The rate of reduction of DHA, by extracts from 2-day-old cotyledons, was almost as great as with extracts from longer-germinated peas (Table 4, cols. 6 and 7) and was equivalent to an electron transfer about three times the total oxygen consumption of the soluble fraction (Table 4, col. 2). As already pointed out, the low oxygen consumption of these extracts from 2-day-old cotyledons is due to the absence of a terminal oxidase. If, however, the oxygen consumption of such extracts is measured after the addition of ascorbic acid oxidase (Table 3a, col. 2), we may calculate, from such data, the percentage of the cyanide-sensitive oxygen consumption which may be ascribed to electron transfer via DHA. The result shows (Table 4, col. 9) that this percentage in the presence of an oxidase would be similar to that found with cotyledons from peas in a more advanced state of germination.

With suspensions from the detached embryo about one-third of the oxygen consumption of the soluble fraction was inhibited by cyanide (Table 4, col. 5), and from the corresponding data on the reduction of DHA in air with cyanide (Table 4, col. 7) nearly all of this could be accounted for by

an electron transfer over the GSH–ascorbic acid system (Table 4, col. 9).

With extracts from the whole embryo the apparent decrease in cyanide-sensitivity of the oxygen uptake of the soluble fraction (Table 3a, col. 5), and the increasing effect of oxygen on the reduction of DHA (Table 3b, col. 4), which were observed during germination, were evidently due to the increasing weight of the plumule + radicle relative to that of the cotyledons.

spondence between levels of respiration of intact tissue and extract being merely fortuitous, but this seems unlikely because experience shows that where serious disorganization of the protoplasm has occurred, respiratory activity is usually grossly affected, ·as was indeed the case with suspensions from the detached embryos. There is the further complication, which may ultimately explain the difference between the cotyledon and detached embryo, namely that with vacuolated tissues the

Table 4. *Effect of cyanide on the oxygen consumption of the soluble fraction of the cytoplasm prepared from suspensions of cotyledons and of detached embryos, and on the rate of reduction of DHA by the same fractions*

Material	O_2 uptake of soluble fraction (μl./g. fresh wt./hr.)				Rate of reduction of DHA (expressed as equivalent of μl. O_2/g. fresh wt./hr.)		Rate in air with KCN as % of that in N_2	Respiration as measured by rate of reduction of DHA in air with cyanide	
	Total	Cyanide-insensitive	Cyanide-sensitive	Inhibition by cyanide (%)	In N_2	In air with KCN		Percentage of cyanide-sensitive respiration of soluble fraction	Percentage of total respiration of soluble fraction
Cotyledons; 10^{-3}M cyanide									
2-Day cotyledon	11	0	11	100	30	30	100	61*	57*
5-Day cotyledon	85	30	55	65	39	39	100	71	46
7-Day cotyledon	93	27	65	71	39	39	100	59	42
9-Day cotyledon	85	25	60	70	36	36	100	60	42
Detached embryos (plumule + radicle); 2×10^{-3}M cyanide									
5-Day detached embryo	64	43	21	33	39	25	64	118	39
7-Day detached embryo	45	21	23	51	30	23	75	100	51
9-Day detached embryo	47	31	16	34	19	13	68	81	28

* Calculated values obtained from rate of reduction of DHA by extract and oxygen consumption of the extract with added ascorbic acid oxidase.

DISCUSSION

We have attempted in this work to determine if the GSH–ascorbic acid enzyme system functions as an electron-transferring system *in vivo*, and if possible to estimate the proportion of the total respiration which passes over it. As in most work of this type, it is relatively easy to construct models of such respiratory systems *in vitro* but more difficult to assess their importance *in vivo*.

In order to obtain a biochemical analysis which provides a reliable assessment of the respiration *in vivo*, the first objective must be to prepare· extracts from the tissue, the respiration of which is a real index of the respiration of the intact tissue. In this work it appears that this was possible in the special case of the cotyledon of the pea seedling. It is, of course, conceivable that even here an artificial respiration is being measured, the corre-

mean substrate concentration in the suspension may be very different from the concentrations of the same substrates in the protoplasm, due to the vacuolar contribution. For these reasons it was impossible to draw any confident conclusions about the part played by the GSH–ascorbic acid system in the respiration of the detached embryo, beyond the suggestion that the system may be operating as an electron carrier *in vivo*.

On the assumption that the oxygen consumption of the extracts from the cotyledon is a reliable index of the respiration *in vivo*, the evidence indicates that a little less than half the respiration of the soluble fraction of the cytoplasm passes over the GSH–ascorbic acid system, which is equivalent to approximately 25 % of the total respiration of the cotyledon.

In a homogeneous solution, one would expect competition between different hydrogen-transfer

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systems for the hydrogen passing over a common coenzyme. In such circumstances the relative affinities of different enzymes, such as TPN-cytochrome *c* reductase, glutathione reductase and diaphorase enzymes would presumably determine the extent of hydrogen transfer over either system. How far this state of affairs exists when two enzymes are localized each in a different part of the cell is difficult to say. The results provided no evidence that the rate of oxygen consumption passing over the GSH–ascorbic acid system was altered by the presence of the cytochrome system localized within the mitochondria. If the respiration of the former system had been affected, one would expect that the oxygen consumption of the soluble part of the cytoplasm, determined separately, would have been greater than when it was present with the mitochondria in the suspension. In fact, the total oxygen consumption of the mitochondria (in the presence of the substrates of the soluble fraction), and of the soluble fraction, when added together, was nearly identical with that obtained before their separation. On the other hand, there was evidence of competition between enzyme systems localized in the soluble part of the cytoplasm in extracts prepared from the detached embryo. Suppression of diaphorase enzymes oxidizing TPNH, by the exclusion of oxygen, appeared to increase the electron transfer over the GSH–ascorbic acid system, as shown by the increased rate of reduction of DHA in nitrogen relative to the rate with cyanide in air.

Apart from ascorbic acid oxidase, which only develops its full activity after the first 3 days of germination, the enzymes associated with the system are present in the dry seed and operate from the early stages of germination. In this early phase the production of GSH, and all that it implies in the activation of SH enzymes, may be of more importance than the functioning of the system in respiration; certainly its full potentiality cannot be realized until development of oxidase activity occurs.

SUMMARY

1. It has been shown with extracts prepared from germinating pea seeds that reduced glutathione and ascorbic acid act as respiratory carriers in an enzyme system which transfers hydrogen to molecular oxygen from substrates of TPN-linked dehydrogenases. The enzymes concerned in this respiratory pathway include dehydrogenase enzymes, glutathione reductase, dehydroascorbic acid reductase and ascorbic acid oxidase.

2. The enzyme dehydroascorbic acid reductase was confined to the soluble part of the cytoplasm and was absent from the mitochondria.

3. The oxygen consumption of suspensions prepared from the cotyledons was of the same order of magnitude as that of the intact tissue, but the oxygen consumption of suspensions from the detached embryo was only about one-tenth that of the intact tissue.

4. The oxygen consumption of suspensions from the cotyledons was almost equally distributed between the mitochondria and soluble fraction of the cytoplasm, but with suspensions from detached embryos the proportion of the respiration attributable to the soluble fraction decreased from 70% at 5 days to 50% after 9 days.

5. Both cyanide and diethyldithiocarbamate, which in millimolar concentrations completely inhibited the oxidation of ascorbic acid, equally depressed the respiration of the soluble fraction of the cytoplasm prepared from suspensions of the whole seedling.

6. Approximately 70% of the total oxygen consumption of the soluble fraction from the cotyledons was cyanide-sensitive and, from the rate of reduction of dehydroascorbic acid by this soluble fraction, it would appear possible that 60% of the cyanide-sensitive respiration might pass over the reduced glutathione–ascorbic acid system. This in turn suggests that 25% of the total respiration of the cotyledons may pass over this route.

7. The reduced glutathione–ascorbic acid system may also function as a respiratory system in the detached embryo but, owing to the lack of correspondence between the respiration of suspensions and intact tissue, no quantitative estimate of its importance can be given.

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Studies in Carotenogenesis

16. THE ACTION OF SOME ANTIBIOTICS, ESPECIALLY STREPTOMYCIN, ON CAROTENOGENESIS IN *PHYCOMYCES BLAKESLEEANUS*[*]

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Bracco & von Euler (1947, 1948) observed that when barley seedlings were germinated on a medium containing streptomycin they produced white coleoptiles and first leaves which contained neither chlorophyll nor carotenoids but normal amounts of anthocyanins. Green seedlings transferred to a streptomycin medium did not lose their plastid pigments, although etiolated seedlings failed to become green or to develop chloroplasts on exposure to light in the presence of streptomycin. These findings have been largely confirmed by using *Pinus jeffreyi* (Bogorad, 1950) and *Pisum* spp. (Schopfer, Bein & Besson, 1952). The green crown gall tumours of carrots also turn white in the presence of streptomycin (Ropp, 1948).

Similar colour changes were first observed in micro-organisms by Provasoli, Hutner & Schatz (1948), using *Euglena gracilis* v. *bacillaris*; in this case there was a permanent loss of chloroplasts, the algae remaining colourless on serial transfer to streptomycin-free media in the light. This has been confirmed by Lwoff & Schaeffer (1949*a*, *b*) and Jirovec (1949); Goodwin & Jamikorn (1954) showed that although considerably reduced by streptomycin, carotenoid synthesis is less affected than chlorophyll synthesis. The whole streptomycin molecule is necessary for inhibition of pigment formation in *E. gracilis* (Provasoli, Hutner & Pinter, 1951) although dihydrostreptomycins A and B are also active (Hutner & Provasoli, 1951). Provasoli *et al.* (1951) also reported no obvious colour change in cultures of five strains of photosynthetic Athiorhodaceae, in which growth had been partly inhibited by streptomycin. Goodwin &

Osman (1953) found, however, in quantitative experiments with *Rhodospirillum rubrum*, that there was a narrow range of concentration over which streptomycin inhibited carotenoid (spirilloxanthin) synthesis to a greater extent than growth; the effect was very much less marked than with *E. gracilis*.

Because of these reports the effect of streptomycin on carotenogenesis in a non-photosynthetic organism, *Phycomyces blakesleeanus*, has been examined in detail. A preliminary report of the inhibition of carotenogenesis in this organism by streptomycin has already been made (Goodwin & Griffiths, 1952) and this has been confirmed by Schopfer, Grob, Besson & Keller (1952).

Although no other antibiotic was found which had the same effect as streptomycin on *E. gracilis* (Hutner & Provasoli, 1951), it has been briefly reported that chloromycetin inhibits carotenogenesis in both pea seedlings and *P. blakesleeanus*, that tetronic acid is active only on pea seedlings and that penicillin is inactive on both organisms (Schopfer, Grob, Besson & Keller, 1952; Schopfer, Grob & Besson, 1952*a*, *b*). In the present investigation these and other antibiotics have also been examined, but in less detail than streptomycin.

EXPERIMENTAL

Cultures. Phycomyces blakesleeanus (−strain) obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland, was used throughout the investigation.

The standard medium was that described by Garton, Goodwin & Lijinsky (1951) modified to contain 2·5% (w/v) glucose. The basal acetate medium used was medium *C* of Friend, Goodwin & Griffiths (1955), based on that of Schopfer & Grob (1950, 1952).

[*] Part 15: Friend, Goodwin & Griffiths (1955).