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Respiration of pea leaf mitochondria and redox transfer between the mitochondrial and extramitochondrial compartment

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In this report, properties of isolated mitochondria from pea leaves have been studied in view to their function in photosynthesis metabolism of a leaf cell. (1) The rates of respiration with various substrates and with a combination of these substrates have been measured with isolated mitochondria. The highest respiration rate was found with NADH, followed by NADPH, malate and glycine. For the oxidation of NADH, NADPH, malate, glycine and 2-oxoglutarate the apparent K_m values were determined. The oxidation of malate and glycine occurred independently of each other as long as electron transport was not limiting. (2) The maximal capacity of mitochondrial ATP synthesis in a leaf was estimated as about 25% of the rate of noncyclic photophosphorylation at maximal rate of photosynthesis. (3) From measurements of NADH/NAD ratios in isolated mitochondria and from previous determinations of the NADH/NAD ratio in the cytosol of spinach leaves it is discussed that in a leaf cell the NADH/NAD ratio in the mitochondrial matrix is higher than in the cytosol. (4) A comparison of the apparent K_m values obtained for the oxidation of external NADH and NADPH with the corresponding concentrations found in the cytosol of spinach leaves suggests that in a leaf cell NADPH is oxidized by mitochondria at a much higher rate than NADH. (5) From the measurement of mitochondrial respiration with glycine and malate as substrates in the presence of a defined malate/oxaloacetate ratio the function of a malate-oxaloacetate shuttle is demonstrated. It is furthermore shown that a malate-aspartate shuttle does not play any significant role in redox transfer under physiological conditions.

Introduction

In photosynthesis of a leaf cell, mitochondria have an essential function in the photorespiratory pathway in oxidizing two molecules of glycine to one serine. An equimolar amount of the NADH produced by this reaction is required for the reduction of hydroxypyruvate to glycerate in the peroxisomes. It has been shown that by a malate/oxaloacetate-shuttle the transfer of redox equivalents from the mitochondria into the peroxisomes is possible [1]. Due to the equilibrium of hydroxypyruvate reduction, the NADH generated in the mitochondrial matrix would be thus sequestrated by hydroxypyruvate reduction unless the redox equivalents required for this reaction are supplied by photosynthetic electron transport, for example by a chloroplastic malate/oxaloacetate-shuttle [2]. Under such conditions the equivalent NADH in the mitochondrial matrix would not be sequestrated by peroxisomal hydroxypyruvate reduction (or cytosolic nitrate reduction) and be available for mitochondrial electron transport. Moreover, redox equivalents released from the chloroplasts could be directly oxidized by the mitochondria via the NADH- or NADPH dehydrogenases located at the outside of the inner mitochondrial membrane [3]. In this way, substrate hydrogen formed by photosynthesis could be used as fuel for mitochondrial oxidative phosphorylation. A cooperation of noncyclic photosynthetic and mitochondrial electron transport seems to be a much more efficient mechanism for the conversion of light energy into ATP than cyclic photophosphorylation [1]. It has been demonstrated that during photosynthesis mitochondrial ATP synthesis has an important function in supplying the cytosol of a leaf cell with ATP. In leaf protoplasts selective inhibition of mitochondrial ATP synthesis by oligomycin resulted in an inhibition of protoplast photosynthesis, accompanied by a decrease of the ATP especially in the cytosol and an apparent

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decrease of sucrose synthesis [4,5]. Mitochondrial oxidation seems not only to be important for an efficient generation of ATP but also for eliminating excessive redox equivalents and thus preventing an overreduction of the carriers of photosynthetic electron transport [5]. Moreover, mitochondria are involved in the provision of carbon skeletons for photosynthetic nitrate assimilation. The mitochondrial tricarboxylic acid cycle has been shown to be operative in green leaves in the light [6].

Whereas in previous years much detailed information about the respiratory properties of plant mitochondria have been gathered (for review see Ref. 7), our knowledge about the coordination of mitochondrial metabolism with the metabolic events in the other compartments of a leaf cell is still fragmentary. Thus it is not fully understood to which extent and in which direction redox equivalents are transferred between the mitochondrial compartment and the other compartments in a photosynthesizing leaf cell, and how metabolic fluxes in the mitochondria are quantitatively related to photosynthesis metabolism. We therefore investigated in the present report the capacity of pea leaf mitochondria to oxidize substrates, to synthesize ATP and to transfer redox equivalents between compartments under conditions resembling the metabolic situation in an illuminated leaf cell. The relation of these mitochondrial capacities to photosynthesis metabolism of a leaf gives an insight into the integration of mitochondrial metabolism in photosynthesis metabolism.

Methods

Pea seedlings (*Pisum sativum* var. Kleine Rheinländerin) were grown in a greenhouse for 12–16 days on soil. The natural light was supplemented by 150 W incandescent lamps. From the pea seedlings mitochondria were prepared essentially after the method of Neuburger et al. [8] with the exception that 0.3 M sucrose instead of 0.3 M mannitol was used throughout the preparation. For details see Ref. 9. The storage medium contained 0.3 M sucrose, 10 mM KH_2PO_4, pH 7.2, 10 mM KCl, 5 mM $MgCl_2$ and 0.1% (w/v) bovine serum albumin (defatted). The intactness of the mitochondria was estimated from the measurement of cytochrome *c*- and ascorbate-dependent O_2-consumption according to Neuburger et al. [8]. The intactness was found to be better than 95% in all preparations.

Respiration was measured in a Clark-type electrode [10] as described by Estabrook [11] in the presence of substrates as indicated after the addition of 0.2 mM ADP (state 3) and after reaching the controlled state (state 4). The incubation medium was the same as the storage medium but supplemented by 0.5 mM NAD. As we observed similar to earlier results [12,13] that the respiration with 2-oxoglutarate and glutamate was enhanced by a factor of 2 when the mitochondria were supplemented with 0.1 mM thiamine pyrophosphate, and that malate oxidation was increased by adding 0.1 mM thiamine pyrophosphate plus 0.05 mM coenzyme A, these cofactors were added in all our respiration measurements. The mitochondrial concentration was 0.08 mg protein/ml incubation medium.

The measurements of NADH and NADPH oxidation by mitochondria were carried out in the same medium as for respiration measurements, and the reaction was monitored by a dual-wavelength photometer (Sigma ZFP 22, Eppendorf, F.R.G.) by measuring the absorbance difference at 334 and 405 nm. The mitochondrial concentration was about 0.002 mg protein/ml. Photosynthesis of pea leaves was measured by an infrared gas analyzer.

Mitochondrial swelling as an indicator for metabolite uptake was measured by the addition of 50 μl mitochondrial suspension (containing 0.5 mg protein) to 1 ml solution containing 5 mM Tes buffer (pH 7.4), 0.1 mM EGTA, 3 μM antimycin A and 100 mM NH_4^+ or K^+ salt of the dicarboxylate indicated. When indicated, 5 mM NH_4^+ phosphate or 10 μM valinomycin were added. Temp 25°C. Immediately after addition of the mitochondria the decrease of absorbance at 546 nm was measured with an Eppendorf photometer.

Results and Discussion

Respiratory properties of pea leaf mitochondria

Mitochondrial respiration was measured by an oxygen electrode with a variety of substrates. If possible, the substrate concentrations were chosen to resemble cytosolic concentrations in a leaf cell. Previous determinations of subcellular metabolite levels in illuminated spinach leaves carried out in our laboratory by nonaqueous fractionation yielded the following estimates of cytosolic concentrations: glutamate 39 mM, aspartate 44 mM, malate 1 mM, 2-oxoglutarate 0.58 mM, NADH 1 μM [14], NADPH 160 μM [15]. In the case of NADH, NADPH and malate higher concentrations had to be applied for the respiration measurements for practical reasons.

Table I shows the respiration rates of a typical mitochondrial preparation. The values shown in brackets are mean values from another series of measurements having been carried out half a year earlier. Both measurements show similar proportions between the respiration rates with the different substrates, with the exception of the rate of glycine-dependent respiration, which in the measurement in brackets is more than 2-fold higher. It has been a general experience that the rate of glycine-dependent respiration varied largely over longer periods. This may be due to the fact that this enzyme is light-induced. It is virtually absent in mitochondria from etiolated plant tissues and is only

TABLE I

Respiration of mitochondria from pea leaves

The values in brackets are mean values of four other series of measurements.

Substrate (mM)	Respiration (natom O/mg protein per min)	
	State 3	State 4
NADH (1)	682 (794)	166
NADPH (1)	411 (414)	144
Glycine (20)	298 (766)	95
Malate (5)	528 (500)	122
Citrate (10)	124 (174)	65
2-Oxoglutarate (1)	257 (278)	86
Glutamate (40)	124 (162)	53
Succinate (10)	331 (366)	122

formed after greening of the plants [16,17]. Apparently the extent of induction of this enzyme is variable. A certain variability was also observed in respect to the respiratory rates with NADH as substrate, amounting in some mitochondrial preparations up to 1200 natom O/mg protein per min. In another series of experiments we measured the concentration dependence of the oxidation of malate, glycine and 2-oxoglutarate. In Table II mean values for the maximal velocity and the apparent K_m have been conferred.

During metabolism the mitochondria are not exposed to a single substrate but a mixture of those listed in Table I. Table III shows respiratory rates which had been determined when two substrates had been added to the mitochondria. The results are from the same experiment as in Table I. For the sake of clarity the respiration rates with NADH, glycine and malate are listed in both tables.

TABLE II

Apparent K_m values for the oxidation of substrates by pea leaf mitochondria (state 3)

Mean value from three experiments (±S.D.).

	K_m (mM)	V_{max} (natom O/mg protein per min)
Malate	2.2 (±1.2)	623 (± 62)
Malate (+40 mM glutamate)	0.63 (±0.18)	558 (± 83)
2-Oxoglutarate	0.09 (±0.06)	240 (± 26)
Glycine	5.2 (±1.0)	456 (±152)

As shown in Table III, the rate of respiration with NADH is not markedly elevated upon the addition of NADPH, malate or glycine as a second substrate. It appears that the rate of the oxidation of external NADH reflects about the maximal capacity of the respiratory chain. The increase in ADP/O ratio, especially observed in the presence of glycine or malate as second substrate indicates that the rate of the oxidation of external NADH had been decreased at the expense of the oxidation of the second substrate (see also Ref. 18).

Glutamate has a dual effect on the mitochondrial oxidation of malate. Whereas a combination of glutamate plus malate yields a lower respiration rate than malate as a single substrate (Table II, III), the corresponding K_m of malate is largely decreased by the presence of glutamate (Table II). It has been shown earlier that the oxidation of malate by plant mitochondria proceeds by parallel action of malic enzyme yielding pyruvate, which is subsequently oxidized to acetyl-CoA, and of malate dehydrogenase producing oxaloacetate as acetyl-CoA acceptor for citrate synthesis [19]. We ascertained the occurrence of this pathway

TABLE III

Respiration of mitochondria from pea leaves in the presence of two substrates

Same preparation and same experiment as in Table I.

Substrate 1	Substrate 2	Respiration (state 3) (natom O/mg protein per min)	ADP/O	ATP synthesis (nmol/mg protein per min)
NADH (1)	–	682	1.24	845
	+NADPH (1)	678	1.27	860
	+glycine (20)	681	1.85	1260
	+malate (5)	695	1.48	1030
Glycine (20)	–	298	2.50	745
	+NADPH (1)	441	1.73	763
	+malate (5)	618	2.52	1560
	+glutamate (40)	302	2.51	758
Malate (5)	–	528	2.21	1170
	+NADPH (1)	621	1.99	1240
	+glutamate (40)	463	2.23	1032

(data not shown) by verifying that the respiration with malate exclusively was stimulated by the addition of thiamine pyrophosphate and coenzyme A and that it was decreased by the addition of bicarbonate, inhibiting malic enzyme activity [19]. The decrease of the apparent K_m for malate is probably caused by a sequestration of oxaloacetate due to transamination [20] and the decrease in respiration due to a prevention of citrate synthesis.

Almost maximal respiration rates are reached when malate and glycine are added together. In the experiment of Figs. 1 and 2 the dependence of respiration with malate and glycine on the concentration of these substrates was determined. In order to avoid an inhibitory effect of oxaloacetate on malate respiration, together with the malate always glutamate at a concentration of 40 mM was added. As shown in Fig. 1, the respiration of malate plus glutamate in addition to the respiration with glycine has about the same apparent K_m for malate as in the absence of glycine. In Fig. 2, for the respiration of glycine in addition to the respiration with malate plus glutamate the apparent K_m for glycine is decreased probably due to the fact that the concentration curve of glycine respiration has been cut off by reaching the ceiling of the maximal respiratory rate. It may be noted that the experiments of Fig.1 and Fig.2 were done with different mitochondrial preparations

TABLE IV

Comparison of the maximal capacities of mitochondrial oxidative phosphorylation and of photophosphorylation in pea leaves

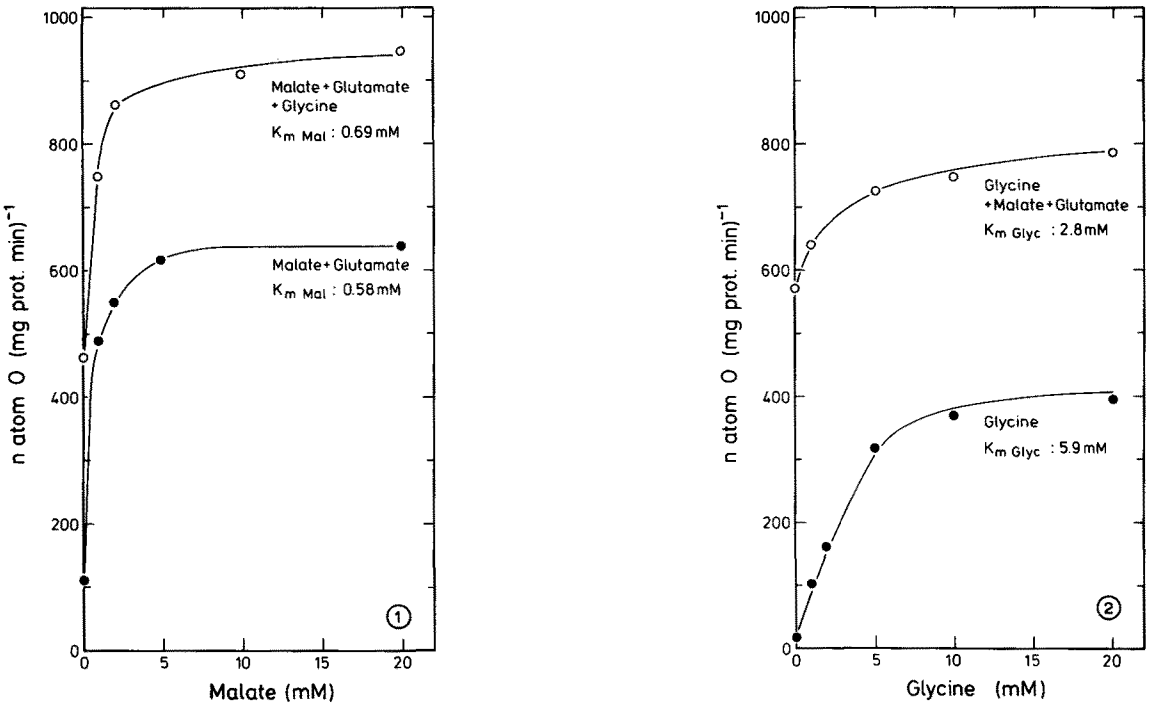
Mitochondrial content, 1.4 mg mitochondrial protein/mg Chl [2].

Oxidative phosphorylation of isolated mitochondria (malate + glycine, see Table III)	126	μmol/mg Chl per h
Photosynthesis of pea leaves (saturating light, 1500 $\mu E\ m^{-1}\ s^{-1}$)	182	μmol O_2/mg Chl per h
Noncyclic photophosphorylation (ATP/2e = 1.33)	484	μmol ATP/mg Chl per h
Oxidative phosphorylation	1.5	μmol/mg protein per min

and therefore the maximal respiration rates are different. It appears that the oxidation of malate and glycine occurs independently until the maximal capacity of mitochondrial electron transport is approached. Earlier results suggested that the oxidation of glycine and malate may occur at different domaines of the mitochondrial matrix [21].

Capacity of mitochondrial ATP synthesis of pea leaves in relation to photosynthesis

By multiplication of the respiration rates with the ADP/O ratios evaluated from the polarographic traces the rates of ATP synthesis have been evaluated (Table III). The highest rates of ATP synthesis are found with



Figs. 1 and 2. Respiration of pea leaf mitochondria in the presence of ADP and P_i (state 3) with malate or glycine. Fig. 1. Dependence on the concentration of malate + 40 mM glutamate ±10 mM glycine. Fig. 2. Dependence on the concentration of glycine ± 5 mM malate + 40 mM glutamate. The experiments of Figs. 1 and 2 were done with different mitochondrial preparations. The apparent K_m values were evaluated by double reciprocal plot. In the presence of glycine or malate as a second substrate, the respiration rate with this substrate alone was subtracted for the determination of the apparent K_m values.

malate plus glycine as substrates, amounting to 1.5 μmol ATP per mg protein per min. From the known content of mitochondria in pea leaves [9] this rate of ATP synthesis is equivalent to 126 μmol ATP per mg chlorophyll per h. A comparison made in Table IV shows that this capacity of mitochondrial ATP synthesis amounts to about one quarter of the rate of noncyclic photophosphorylation at maximal rate of photosynthesis in pea leaves.

Redox gradient between the NADH / NAD systems in the mitochondrial matrix and the cytosol

By nonaqueous subcellular metabolite analysis of illuminated spinach leaves the cytosolic NAD concentration has been determined to be about 0.5 mM [15]. From this value and the determination of the cytosolic levels of glutamate, aspartate, 2-oxoglutarate and malate the cytosolic NADH/NAD ratio has been evaluated on the reasonable assumption that the reactions catalyzed by the cytosolic malate dehydrogenase and glutamate-oxaloacetate transaminase are near to equilibrium. This calculation yielded for illuminated spinach leaves a cytosolic NADH/NAD ratio of $1.2 \cdot 10^{-3}$ corresponding to a NADH concentration of 0.6 μM [14,15]. This value is similar to the K_m of spinach nitrate reductase for NADH (1.4 μM [22]) and also similar to the NADH/NAD ratio found in animal tissues such as liver [23]. Since it has not yet been possible to determine the mitochondrial NADH/NAD ratio in intact leaves, we had to rely on measurements with isolated mitochondria. Table V shows the measurement of the redox state of the endogenous NADH/NAD system in pea leaf mitochondria during respiration with glycine as substrate. The values obtained are similar to results of earlier measurements with animal mitochondria [23] and mitochondria from potatoe tuber and pea leaves [24]. It appears from these results that like in animal tissues also in plant cells the NADH/NAD system in the matrix is more reduced than that in the cytosol. Provided that in a leaf the mitochondrial NADH/NAD ratio is in a state intermediate between state 3 and state 4 measured in the isolated mitochondria, the redox gradient between the NADH/NAD in the mitochondrial matrix and the cytosol would be in the order of 100. Considerations about a redox transfer between the cytosol and the mitochondria have to account for this.

TABLE V

Redox state of endogenous pyridine nucleotides during respiration of pea leaf mitochondria with glycine

Mean values from three experiments (± S.D.).

	State 3	State 4	
Respiration	512 ± 60	101 ± 23	natom/mg protein per min
NADH	0.31 ± 0.08	1.24 ± 0.23	nmol/mg protein
NAD	4.72 ± 0.37	3.79 ± 0.27	nmol/mg protein
NADH/NAD	0.065 ± 0.020	0.33 ± 0.06	

External pyridine nucleotides as substrates of mitochondrial electron transport

Direct oxidation of external NADH constitutes a way for transferring redox equivalents from the cytosol to the mitochondria despite of the existing redox gradient. Plant mitochondria, in contrast to animal mitochondria, are able to oxidize external NADH and NADPH (for review see Ref. 25). NADH is usually oxidized faster than other substrates. Available evidence suggests that NADH and NADPH are oxidized by two different dehydrogenases [25] having in common that they are bypassing the first coupling site. Apparent K_m values for the oxidation of external NADH were found in the range between 10 and 100 μM, depending on the measuring conditions and the origin of the mitochondria, the apparent K_m values for the oxidation of external NADPH being generally higher than those for NADH [25].

For the respiration measurements in Tables I and III for practical reasons unphysiologically high concentrations of NADH and NADPH had been employed. For an estimation of the rate of external NADH and NADPH oxidation under *in vivo* conditions, we determined the substrate dependence of NADH and NADPH oxidation in the presence of 0.5 mM NAD and 0.1 mM NADP (resembling the cytosolic NAD and NADP concentrations in a spinach leaf [15]). These experiments were done with very low concentrations of mitochondria in a cuvette of a dual-wavelength photometer allowing the measurement of NAD(P)H absorbance at 334 nm with 50 fold scale expansion. In this way the concentration dependence of NAD(P)H oxidation could be determined very accurately. Fig. 3 shows a double-reciprocal plot of such an experiment. Table VI presents mean values of the apparent K_m values assayed in the absence and presence of NAD and NADP, respectively. In the case of NADH oxidation, the addition of 0.5 mM NAD had no effect on K_m and V_{max}. The V_{max} of the NADPH oxidation was not affected by NADP, too, but the K_m was increased by a factor of 2. The effect of other respiratory substrates on the oxidation of NADH and NADPH was also investigated (Table VII). The V_{max} of the NADH oxidation was decreased upon the addition of glycine, malate and 2-oxoglutarate to an extent similar to the respiration rates found with these substrates in Table I. The results of Table III have shown that under saturating conditions the oxidation rate of external NADH is about equal to the maximal capacity of the mitochondrial respiratory chain. It appears from the results of Table VII that the oxidation of glycine, malate and 2-oxo-

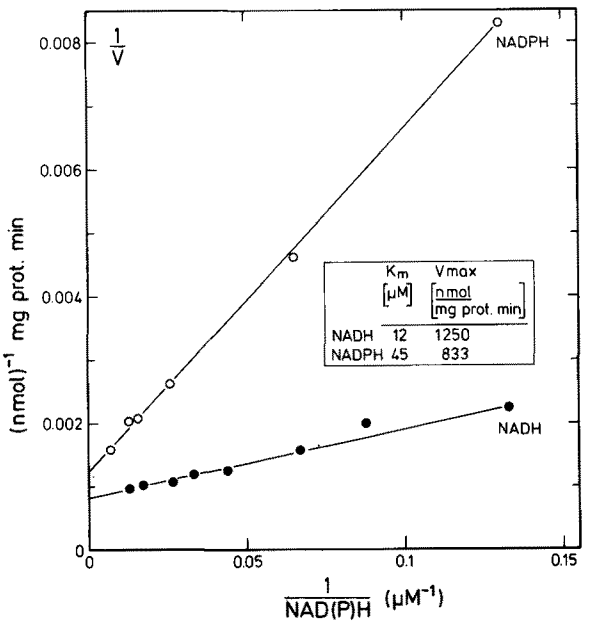


Fig. 3. Double-reciprocal plot of the concentration dependence of the oxidation rate of NADH and NADPH of pea leaf mitochondria in the presence of ADP and P_i (state 3).

TABLE VI

Kinetic constants for the oxidation of external pyridine nucleotides by mitochondria from pea leaves in the presence of 1 mM ADP, 10 mM P_i and 2 mM $CaCl_2$

Spectrophotometric assay. Mean values from three experiments.

Substrate	Addition (mM)	Oxidation of NAD(P)H	
		K_m (μM)	V_{max} (state 3) (nmol/mg protein per min)
NADH	–	12	925
NADH	NAD (0.5)	13	924
NADPH	–	16	520
NADPH	NADP (0.1)	33	513

TABLE VII

Effect of respiratory substrates on the oxidation of external pyridine nucleotides by mitochondria from pea leaves in the presence of 1 mM ADP and 10 mM P_i (state 3)

The assay contained 2 mM Ca^{2+}, 0.1 mM TPP and 0.05 mM CoASH. Spectrophotometric assay.

Substrate	Addition (mM)	Oxidation of NAD(P)H	
		K_m (μM)	V_{max} (nmol/mg protein per min)
NADH + NAD (0.5)		17	1191
	glycine (10)	11	825
	malate (5)	12	818
	2-oxoglutarate (1)	15	945
NADPH + NADP (0.1)		26	618
	glycine (10)	27	489
	malate (5)	27	527
	2-oxoglutarate (1)	22	631

glutarate competes favourably with the oxidation of external NADH for the capacity of the respiratory chain. In the presence of these substrates the substrate saturation curve of NADH oxidation probably reaches the ceiling of the maximal capacity of mitochondrial respiration, explaining the decrease in K_m observed under these conditions. A decrease of NADH oxidation when glycine was present as second substrate has been earlier observed by Bergman and Ericson [18]. Glycine and malate also decrease the maximal rate of NADPH oxidation, although the extent of this decrease is smaller than observed with NADH oxidation (Table VII). 2-Oxoglutarate did not show any marked effect on NADPH oxidation. Since the maximal rate of NADPH oxidation is only about half of the maximal capacity of mitochondrial respiration, a competition of NADPH with these other substrates for being oxidized by the respiratory chain may be not so strong, at least in state 3 where this measurements were done.

Subcellular metabolite analysis of illuminated spinach leaves had yielded for NADH and NADPH cytosolic concentrations of 0.6 μM and 160 μM respectively [15]. On the assumption of simple Michaelis-Menten kinetics the rates by which NADH and NADPH would be oxidized by the mitochondria can be calculated using the above concentrations according to the equation: $V = V_{max} \times S/S + K_m$. This calculation yields the following oxidation rates:

NADH oxidation:	43	nmol/mg protein per min
	3.6	μmol/mg Chl per h
NADPH oxidation:	418	nmol/mg protein per min
	35	μmol/mg Chl per h

These results illustrate that in the presence of the cytosolic substrate concentrations found in spinach leaves in pea leaf mitochondria the rate of NADPH oxidation would be almost ten times higher than that of NADH oxidation. It may be noted that the measurements of Tables VI and VII have been carried out in the presence of Ca^{2+} ions. The oxidation of external NADH by mitochondria requires about 1 μM free Ca^{2+} ions for activity [3], and Ca^{2+} ions are also needed for NADPH oxidation. Furthermore, the NADH- and NADPH dehydrogenases were found to differ in their pH optima [26]. In spinach leaf mitochondria the oxidation of NADH was found to be maximal at pH 7.0 and that of NADPH oxidation at pH 6.0, with only very low activity at pH 7.0 (K_m NADPH 1 mM, V_{max} 36 nmol/mg protein per min) [26]. As shown above, in pea leaf mitochondria the corresponding K_m and V_{max} values, determined at pH 7.2, are very different. A high rate of NADPH oxidation by pea leaf mitochondria at neutral pH have been observed earlier [27]. Apparently, mitochondria from pea and spinach leaves differ

markedly in regard to NADPH oxidation. Differences in the ability of leaf mitochondria from various plants species to oxidize external NADPH have been also reported by Arron and Edwards [28]. It remains to be elucidated to which extent the oxidation of external NADH and NADPH may be regulated by changes in pH and in the free Ca^{2+} ion concentration in the cytosol.

Redox transfer by metabolite shuttles

The redox gradient between the NADH/NAD systems in the mitochondrial matrix and the cytosol would make a transfer of redox equivalents from the mitochondrial matrix into the cytosol possible. In principle such a transfer might be achieved by a shuttle of malate and oxaloacetate or by a shuttle of malate, aspartate, 2-oxoglutarate and glutamate. Mitochondria from spinach and pea leaves were shown to contain a specific translocator for oxaloacetate with a very high affinity to its substrate (K_m 3.7 μM). This translocator is suited to catalyze an efficient malate-oxaloacetate shuttle under physiological conditions [9]. In plant leaf mitochondria transport of malate and oxaloacetate is so rapid that its kinetics cannot be resolved by silicon oil layer filtration experiments. Measurements of swelling in isoosmolar concentration of dicarboxylates revealed that oxaloacetate and malate are very rapidly taken up by electrogenic uniport [29]. As shown in Fig. 4, pea leaf mitochondria contained in a medium of potassium malate swell rapidly upon the addition of valinomycin. Similarly, a very high permeability in the presence of valinomycin is also found for the potassium salts of glutamate and aspartate, indicating that all these substances can be transported by electrogenic uniport (Fig. 4). Although at lower rate, glutamate and aspartate seem also to be transported by counterexchange with phosphate, as revealed from the swelling of the ammonium salts in the presence of phosphate [30]. 2-

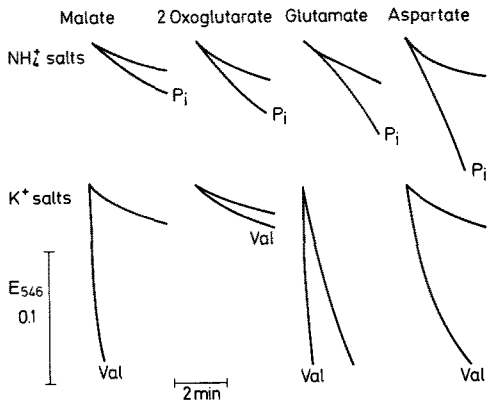


Fig. 4. Swelling of pea leaf mitochondria in NH_4^+ or K^+ salt of various dicarboxylates (100 mM) as indicator for metabolite uptake. When indicated, 5 mM NH_4^+ phosphate or 10 μM valinomycin were added. For details see Methods.

TABLE VIII

Effect of 2-oxoglutarate + aspartate on state 3 respiration of pea leaf mitochondria with malate and glycine as substrate

Additions (mM)	Respiration (natom O/mg protein per min)	
	glycine 20 mM	malate 5 mM
Control	316	479
+ Asp (40)	304	476
+ 2-OG (2)	505	549
+ Asp (40) + 2-OG (2)	345	445
± Asp in presence of 2-OG	160	104

Oxoglutarate, in contrast to malate, glutamate and aspartate, causes only little swelling in the presence of phosphate and hardly any with valinomycin. Thus 2-oxoglutarate appears to be transported across the inner mitochondrial membrane by electric uniport at a much lower rate than malate, glutamate and aspartate.

With pea leaf mitochondria a redox transfer by malate-aspartate shuttle, although occurring to only limited extent, and only under extreme conditions, can be demonstrated. In the experiment of Table VIII it is shown that the respiration with glycine or malate as substrate is virtually unaffected by 40 mM aspartate, but is stimulated by 2-oxoglutarate as a second substrate. The stimulation is reversed when aspartate and 2-oxoglutarate are added together. This effect, which has been often reproduced, seems to be due to the formation of oxaloacetate from aspartate and 2-oxoglutarate, as catalyzed by mitochondrial glutamate-oxaloacetate transaminase, sequestrating internal NADH via malate dehydrogenase. Phthalonate, a strong inhibitor of mitochondrial oxaloacetate transport did not reverse this effect, excluding that the oxaloacetate was formed in the medium by contaminating enzymes (data not shown).

The experiment of Table VIII reflects an extreme metabolic situation as there was no glutamate added and the oxaloacetate concentration in equilibrium with the added aspartate and 2-oxoglutarate is very high. In order to simulate more physiological conditions, respiration was measured with 10 mM glycine plus 5 mM malate in the presence of various concentrations of oxaloacetate (Fig. 5). To maintain a steady state concentration of oxaloacetate, it was not added as such, but generated from glutamate plus aspartate plus 2-oxoglutarate via glutamate oxaloacetate transaminase. For this purpose the concentrations of glutamate and aspartate in the medium were varied to yield a certain equilibrium concentration of oxaloacetate (see legend to Fig. 5). Upon the addition of 2-oxoglutarate and glutamate-oxaloacetate transaminase (GOT) a defined malate-oxaloacetate ratio was produced in the medium. This enabled to study the operation of the

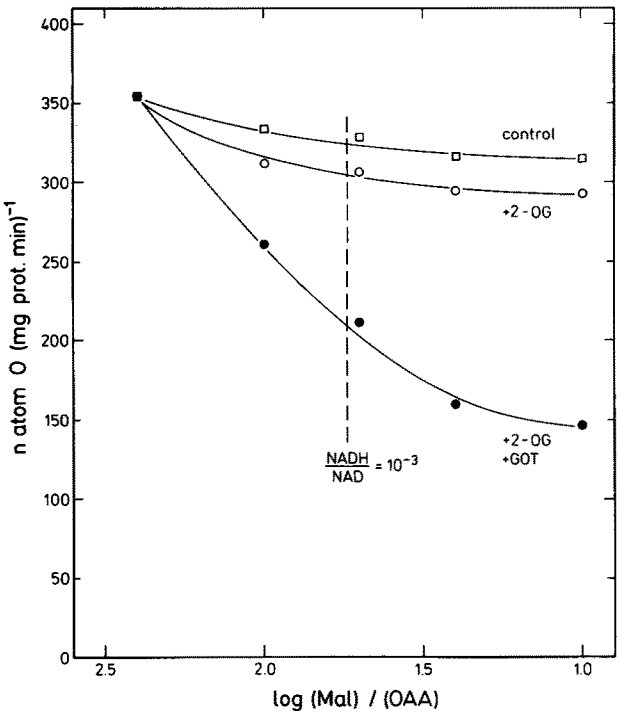


Fig. 5. Measurement of respiration in the presence of ADP and P_i (state 3) with malate (5 mM) plus glycine (10 mM) as substrates. The incubation medium contained (from left to right) concn. in mM: Asp (10), Glu (45); Asp (25), Glu (45); Asp (40), Glu (40); Asp (40), Glu (18); Asp (40), Glu (7). After addition of mitochondria (0.1 mg prot/ml) 0.6 mM 2-oxoglutarate and 0.06 μkat glutamate oxaloacetate transaminase were added as indicated. The respiration rate was determined after a steady state had been reached.

malate/oxaloacetate shuttle under defined conditions. In a second series of experiments 2-oxoglutarate without GOT was added to the medium. Under these conditions glutamate, aspartate and 2-oxoglutarate would have to enter the mitochondria for the equilibrium concentration of oxaloacetate to be generated via the glutamate-oxaloacetate transaminase in the matrix. This experiment was designed to decide whether a malate-aspartate shuttle is operating at the defined metabolic conditions simulated in the reaction.

In Fig. 5 the respiration rates measured in the two experimental series have been plotted against the logarithm of the malate-oxaloacetate ratios. In the series where GOT was added to the medium an increase of the malate-oxaloacetate ratio results in a large decrease of the respiratory rate, illustrating the sequestration of the NADH generated from glycine and malate oxidation by oxaloacetate. These results clearly show that a malate-oxaloacetate shuttle is operative under physiological conditions. In the absence of GOT in the medium, there is only a slight decrease of the respiratory rate observed upon the addition of 2-oxoglutarate. This result demonstrates that under physiological conditions a malate-aspartate shuttle does not have any marked function in transferring redox equivalents from the matrix of pea leaf mitochondria to the cytosol. This conclusion is in agreement with results of Dry et al. [31].

As shown in Fig. 5, the malate-oxaloacetate ratio being in equilibrium via malate dehydrogenase with a ratio NADH/NAD = 10^{-3} (marked by a hatched vertical line) decreased the respiration rate by about one third. This result shows that in the presence of a ratio NADH/NAD = 10^{-3} as found in the cytosol of illuminated spinach leaves [14], mitochondrial respiration, although being controlled, can operate at a considerable rate. Assuming an ADP/O of 2.5, the residual respiration with glycine plus malate as substrate would enable an ATP synthesis rate of 42 μmol/mg Chl per h, which is still about 10% of the rate of noncyclic photophosphorylation at maximal photosynthesis of pea leaves (see Table IV).

Concluding remarks

The pea leaf mitochondria studied in this report showed in average a maximal respiratory rate of about 800 natom O/mg protein per min. With a mitochondrial content of pea leaves equivalent to 1.4 mg mitochondrial protein per mg chlorophyll [9], the respiratory capacity of pea leaves can be thus evaluated as 67 μatom O/mg Chl per h. Respiration measurements with pea leaves, yielded an average respiration rate of 24 μatom O/mg Chl per h. This respiration rate is far below the capacity of mitochondrial respiration and ATP synthesis. A large portion of the mitochondrial capacity may be therefore regarded as a reserve to cope with extreme metabolic situations.

Different modes of redox transfer between the mitochondrial and extramitochondrial compartments have been dealt with. Evidence has been presented that a malate-oxaloacetate shuttle operates under simulated physiological conditions. The high capacity of the mitochondrial malate-oxaloacetate shuttle reported previously [9] and our finding that mitochondrial respiration continues to a large extent in the presence of an external malate/oxaloacetate ratio which is in equilibrium with a ratio NADH/NAD = 10^{-3} suggests that despite the very active malate-oxaloacetate shuttle a redox gradient between the mitochondrial matrix and the cytosol is maintained. Such a difference in gradients might be achieved by a control of malate transport by oxaloacetate, as reported earlier [29].

As the malate-oxaloacetate shuttle seems only suited for the export of redox equivalents from the mitochondria, e.g. for hydroxypyruvate reduction in the peroxisomes or nitrate reduction in the cytosol, an oxidation of external pyridine nucleotides would require the external NADH and NADPH dehydrogenases. Although the regulation of these dehydrogenases is not fully understood, our results suggest that in the steady state of photosynthesis the external NADH dehydrogenase, though being the most active in the

mitochondria, does not play a major role in redox transfer. As the apparent K_m of the external NADH dehydrogenase is much higher than the estimated NADH concentration in the cytosol, even with the fully activated enzyme, the rate of NADH oxidation in the leaf should be rather low. This may be important to prevent that the redox equivalents transported out of the mitochondria by a malate-oxaloacetate shuttle are reoxidized by the external NADH dehydrogenase. In this way the first coupling site of ATP synthesis of the respiratory chain would be circumvented which would result in a loss of energy for the plant cell. It seems therefore that in a plant cell the external NADH dehydrogenase will be only operative at larger scale when the cytosolic NADH/NAD system is excessively reduced, e.g. by redox equivalents arising from photosynthetic electron transport.

Because of the high cytosolic NADPH concentration, at least in pea chloroplasts the major route for the oxidation of external redox equivalents by the mitochondria may be the external NADPH dehydrogenase. For this the NADPH could be delivered from the chloroplasts by a triosephosphate-phosphoglycerate shuttle, coupled in the cytosolic compartment with the nonphosphorylating, irreversible glyceraldehyde phosphate dehydrogenase catalyzing the conversion of glyceraldehydephosphate and NADP to 3-phosphoglycerate and NADPH [32].

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