Plant Physiol. (1987) 83, 349–353
0032-0889/87/83/0349/05/$01.00/0

The Simultaneous Determination of Carbon Dioxide Release and Oxygen Uptake in Suspensions of Plant Leaf Mitochondria Oxidizing Glycine[1]

Received for publication June 4, 1986 and in revised form October 13, 1986

ROSS MCC. LILLEY*,[2,3] HOLGER EBBIGHAUSEN, AND HANS W. HELDT
Institut für Biochemie der Pflanze, Universität Göttingen, Untere Karspüle 2, 3400 Göttingen, Federal Republic of Germany

ABSTRACT

The construction and operation of a device for continuous measurement of CO_2 release by suspensions of respiring mitochondria is described. A combination of this device with a Clark-type O_2 electrode was used for simultaneous measurement of respiration and of CO_2 release by spinach and pea leaf mitochondria with glycine as substrate. Both mitochondrial preparations showed high rates of respiration and high respiratory control ratios. The addition of oxaloacetate not only inhibited O_2 uptake substantially, but also greatly stimulated glycine oxidation as monitored by CO_2 release. In spinach leaf mitochondria, the maximal rates of glycine oxidation thus obtained, were two times higher than the rate of glycine oxidation required at average rates of photorespiration. It is concluded from these results that under saturating conditions the capacity of glycine oxidation by intact mitochondria exceeds the capacity of glycine-dependent respiration.

Respiration in suspensions of isolated mitochondria is customarily determined by following O_2 concentration changes with a polarographic O_2 electrode. This is a most useful way of measuring the activity of the respiratory electron transport chain, but does not reflect the rates of preceding mitochondrial respiratory events, such as substrate decarboxylation, when these are coupled with other redox processes apart from electron transport to molecular oxygen. Thus, in isolated plant mitochondria, the oxidation of glycine can be coupled with the reduction of OAA[4] to malate without electron transport occurring (6, 17). As this is an important partial reaction in the photorespiratory pathway, there is a need to measure the rate of glycine oxidation independently from electron transport. Since the oxidative conversion of two molecules of glycine to one serine yields one CO_2 and one NH_3, the radioactive assay of ^{14}C release from [^{14}C] glycine (14, 17) and the measurement of NH_4^+ release by an NH_4^+ electrode (2, 3) have been employed for monitoring glycine oxidation. We present here an alternative approach based on the determination of CO_2 by a CO_2 electrode, a method which can also be used for other processes connected with CO_2 release, *e.g.* citric acid cycle turnover.

The CO_2 electrodes used widely for the clinical determination of blood CO_2 partial pressure normally operate in the range of 8 to 400 mm P_{CO_2}. Because about 80% of dissolved carbon in neutral solutions is present as HCO_3^-, the concentration of CO_2 in mitochondrial suspensions (typically around pH 7.4) is much lower than this. The lower limit of sensitivity (8 mm P_{CO_2}) corresponds to a dissolved CO_2 concentration of 0.24 mM, and a total (CO_2 + HCO_3^-) concentration of 4.8 mM. Because of this comparatively low sensitivity, these electrodes have not been used with mitochondrial suspensions.

For monitoring CO_2 release by perfused organs, such as heart, the technique has been increased in its sensitivity by acidifying the measuring sample and thus converting all bicarbonate into CO_2 (12). In the present report this method has been further refined to an extent that it enables CO_2 measurements to be performed on diluted mitochondrial suspensions with sensitivity similar to O_2 electrode measurements. The application of this method for studying the oxidation of glycine by spinach and pea leaf mitochondria has revealed that the capacity of these mitochondria to oxidize glycine exceeds the capacity of glycine-dependent respiration.

MATERIALS AND METHODS

Determination of CO_2. A CO_2 electrode (Radiometer A 5037) was mounted inside an aluminum jacket (Fig. 1A), with the silicone membrane forming one side of a measuring flow chamber (*C*—volume 40 mm^3) cut in a Perspex block (*P*). The inflow of acidified mitochondrial reaction mixture to the chamber was through a length of stainless steel tubing to the bottom of the measuring chamber, while the outflow left the chamber at the top (Fig. 1B). This ensured that any air bubbles in the inflow did not accumulate in the measuring chamber. The Perspex block was sandwiched between the jacket and a second aluminum block, both of which contained internal channels, through which constant temperature water was pumped for temperature regulation. The electrode potential (mV) was measured by a Metrohm pH meter and recorded on a chart recorder. The conversion of recorded potentials to CO_2 concentrations was done subsequently using a calibration graph prepared on the day of the experiment by measuring the potentials of a series of reaction mixtures of known CO_2 concentration.

Simultaneous Measurement of O_2 Consumption and CO_2 Release. The mitochondrial reaction mixture was made up in a potentiometric O_2 electrode assembly (Hamsatech, King's Lynn, UK) operated and calibrated essentially as described by Delieu and Walker (4). The electrode output was recorded via a second pen on the chart recorder. The standard reaction vessel plunger

[1] Supported by the Deutsche Forschungsgemeinschaft.

[2] Present address: Department of Biology, University of Wollongong, P.O. Box 1144 Wollongong, N. S .W. 2500, Australia.

[3] Supported by an Alexander von Humboldt Foundation Fellowship.

[4] Abbreviations: OAA, oxaloacetic acid; AA, antimycin A; RCR, respiratory control ratio.

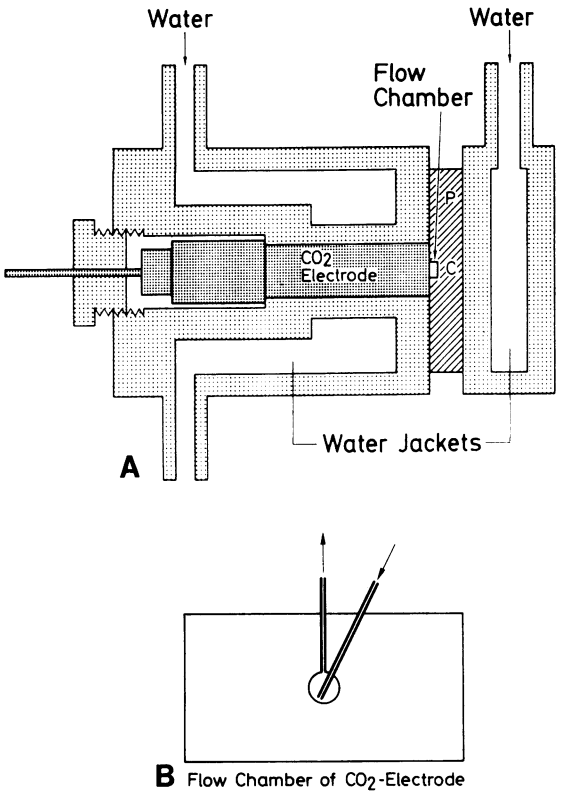

FIG. 1. A, Temperature-controlled jacket and measuring chamber for CO_2 electrode. B, Cross-section of measuring chamber.

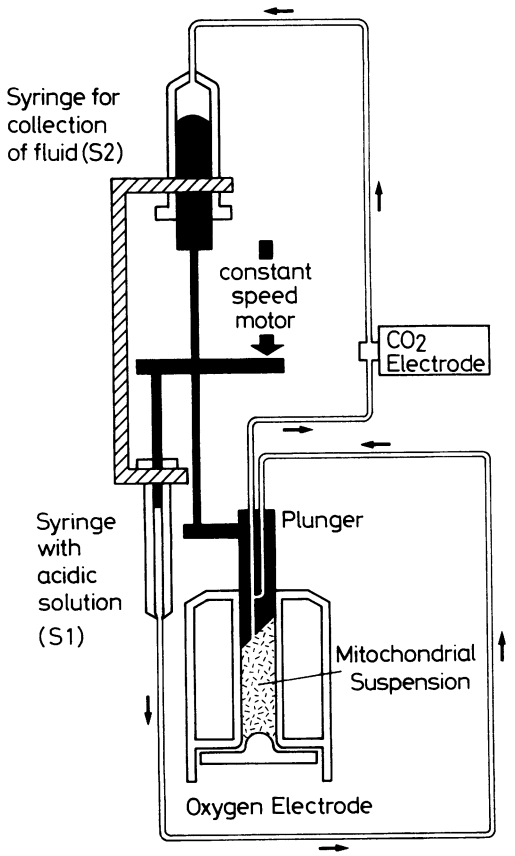

FIG. 2. Combined syringe pump assembly.

was replaced by a plunger (Fig. 2) mounted on an assembly driven by a single constant speed motor. This assembly connected the downward movement of the plunger with the closure of syringe S1 and the opening of syringe S2. Mitochondrial suspension was withdrawn from the O_2 electrode chamber at a flow rate of 1.6 $\mu l\ s^{-1}$ by the opening syringe. Simultaneously, an acidic solution (2.5% phosphoric acid, 0.04% bromothymol blue) was pumped from the closing syringe (S1-Hamilton, 500 μl) at a flow rate of 0.16 $\mu l\ s^{-1}$. The acidic solution met the effluent from the O_2 electrode chamber at a junction within the plunger (Fig. 3). In this way, the withdrawn mitochondrial suspension was acidified to about pH 1 in order to convert all bicarbonate into CO_2 before entering the CO_2 electrode chamber. The flows were such that the acidic solution could not reach the mitochondrial suspension in the O_2 electrode chamber. As a safeguard, the bromothymol blue present in the acid allowed visual confirmation that the proper flows and mixing were occurring. The initial volume of the reaction mixture was 2.5 ml, and the volume at the end of the experiment was about 0.6 ml, giving a duration of about 19 min for the experiment.

Preparation of Mitochondria. Spinach (*Spinacia oleracea*) and pea (*Pisum sativum*) were grown hydroponically in a glasshouse with supplementary lighting, or in a growth cabinet. Mitochondria were prepared from deribbed spinach leaves or from pea seedling shoots at the stage of leaf expansion as described by Ebbighausen *et al.* (7).

Experimental Procedure. The reaction mixture (total volume 2.5 ml) contained 0.3 M sucrose, 10 mM K-phosphate, 10 mM KCl, 5 mM $MgCl_2$, 0.1% defatted BSA, and mitochondria containing 0.1 to 0.6 mg protein (pH 7.4) plus, typically, 0.5 mM NAD^+ and ADP as indicated. After making up the reaction mixture, the tubing connections were made to the plunger and the plunger was inserted into the apparatus. Initially, the tubing for delivering acid was filled only to about 10 mm from the delivery end. On starting the syringe drive, 10 to 15 s elapsed before acid began to mix with the mitochondrial solution flowing through the plunger. This ensured that there was no possibility of acid diffusing into the reaction chamber before the pump was started and the flows established.

The addition of substrates and other components to the reaction vessel was done with microsyringes, inserting the needle down the groove in the plunger (Fig. 3). Additions were made at

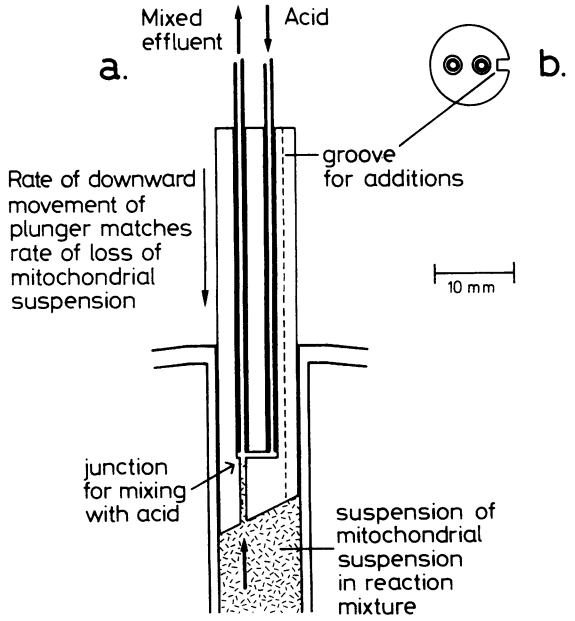

FIG. 3. Section through O_2 electrode plunger (a). Cross section (b).

precise times, corresponding to known volumes of the reaction mixture, calculated beforehand from the known rate of removal of solution from the reaction vessel.

RESULTS AND DISCUSSION

Method. A typical calibration graph, obtained by placing bicarbonate solutions of known concentrations in place of the mitochondrial reaction mixture, is shown in Figure 4. The electrode output was essentially a negative linear function of the logarithm of the bicarbonate concentration. Electrodes of this type can be affected by variations in temperature, hydrostatic pressure, and osmotic pressure of the solution being measured. No significant variations in output occurred due to these factors.

The CO_2 electrode did not respond significantly to the presence of NH_4^+ in the mitochondrial reaction mixture *e.g.* the addition of $(NH_4)_2SO_4$ to the reaction chamber to a concentration of 1 mM had no effect on the electrode output. The lack of interference by NH_4^+ ions is important since ammonia is a product of glycine oxidation. The electrode did respond to millimolar concentrations of cyanide, and the use of this inhibitor was avoided. Apparently, volatile weak acids, formed on acidification of the medium, are capable of interfering with CO_2 measurements. When OAA was present in the reaction vessel, CO_2 was released at a low rate due to nonenzymic decarboxylation (10) in the acidified reaction mixture. The rate of nonenzymic decarboxylation was determined as 1 μM min^{-1} when the reaction medium containing 500 μM OAA was present in the reaction chamber, and CO_2 release rates measured in the presence of added OAA were corrected for this.

The lag time between a step change in (CO_2 + HCO_3^-) in the mitochondrial reaction vessel (achieved by adding bicarbonate to the vessel) and the initiation of the response by the CO_2 electrode was 25 s, due to the dead space in the tubing. The response time of the electrode was about 2 min from the initiation to 100% response, due to a combination of the time to flush out the measuring chamber and the inherent response time of the electrode.

Simultaneous Measurements of Respiration and CO_2 Release by Leaf Mitochondria. With spinach leaf mitochondria oxidizing glycine, O_2 uptake and CO_2 release were measured. Some original recorder traces of simultaneous O_2 and CO_2 electrode recordings are shown in Figure 5. The mitochondria exhibited highly coupled electron transport. From the O_2 trace the respiratory control ratio after the first addition of ADP was 8.0, which increased

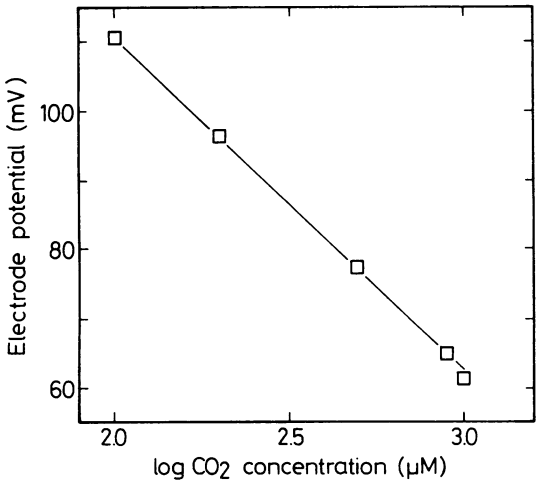

FIG. 4. Calibration graph of the CO_2 electrode. The corresponding concentrations of CO_2 were added as bicarbonate solutions in place of the mitochondrial suspensions in the O_2 electrode vessel.

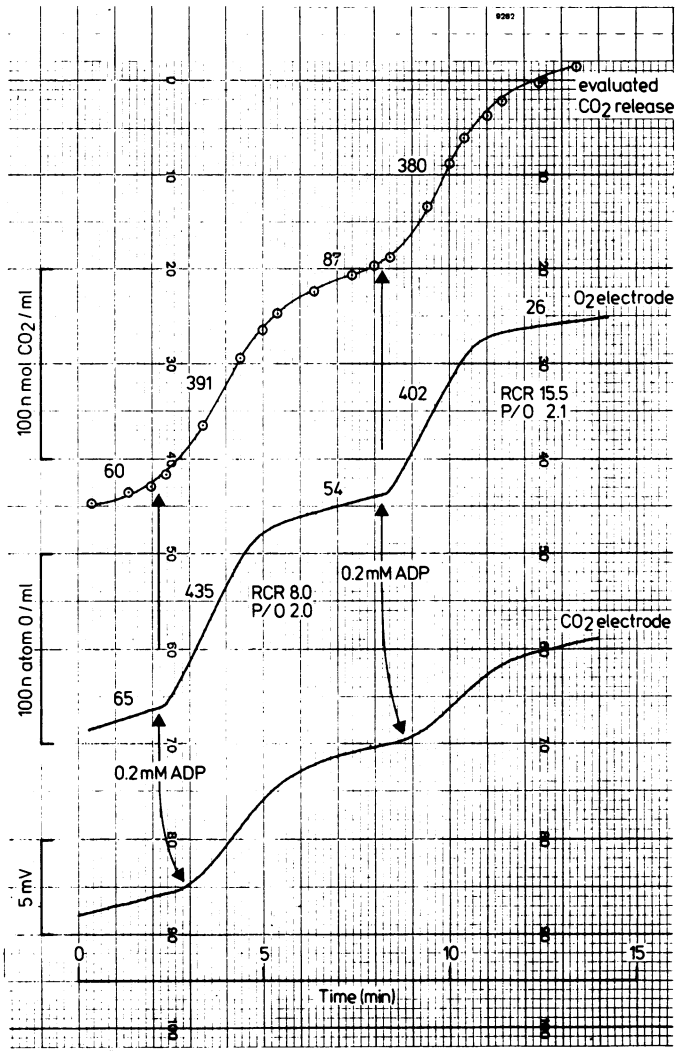

FIG. 5. Original traces of simultaneous measurement of CO_2 release and O_2 consumption by suspensions of isolated spinach leaf mitochondria with 20 mM glycine as substrate in a total volume of 2.5 ml. For details see "Materials and Methods." The concentration of mitochondria was 0.23 mg protein/ml in Figure 5 and 0.16 mg protein/ml in experiments of Figures 6 and 7 (p. 352). ADP, OAA, and AA were added as indicated.

after the second addition of ADP to 15.5. Similar increases of respiratory control ratios after multiple additions of ADP have been observed earlier in our laboratory (7). The CO_2 electrode trace lagged behind the O_2 trace, due to the dead space in the tubing and the CO_2 electrode chamber, as discussed before. Using a calibration curve analogous to Figure 4, the release of CO_2 was evaluated from the CO_2 electrode trace, corrected for the lag time and plotted above the recorder traces (Fig. 5). In the presence of ADP the release of CO_2 paralleled the uptake of O_2 with a CO_2/O_2 quotient of 0.90 to 0.95 which is close to the expected stoichiometry of 1. The higher rate of CO_2 evolution, in relation to the rate of O_2 consumption observed when respiration is decreased after running out of ADP, represents largely an overestimation due to the dead space and response lag of the CO_2 measuring system. For an accurate determination of a very low rate of CO_2 evolution following a very high one, a measuring time of about 6 to 8 min would be required. In the experiment of Figure 6, mitochondria were allowed to oxidize glycine in the presence of an excess of ADP. The addition of 500 μM OAA to these mitochondria caused a strong inhibition of O_2 uptake, whereas the CO_2 release was enhanced by more than 50%. In a

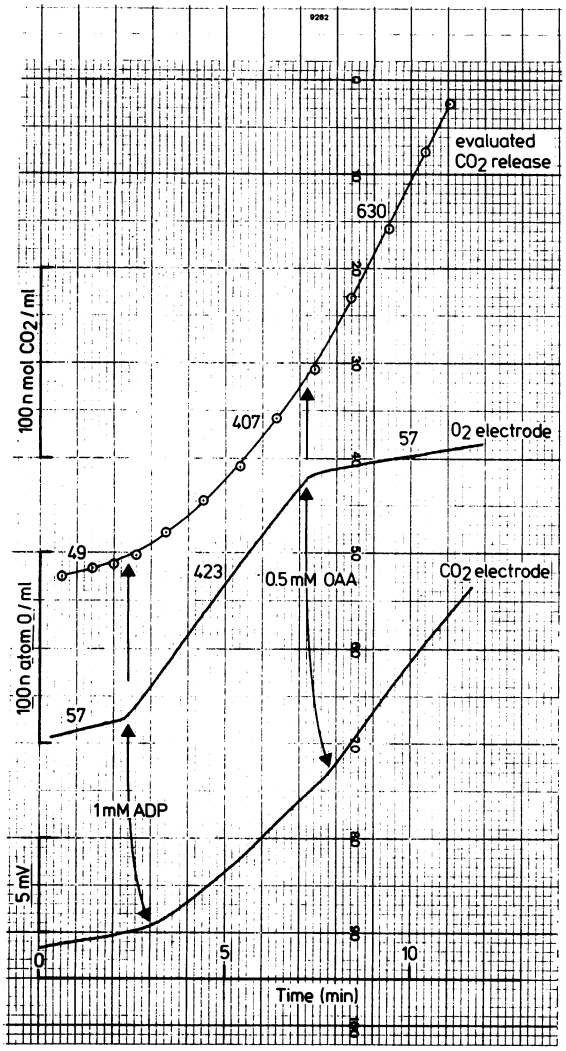

FIG. 6

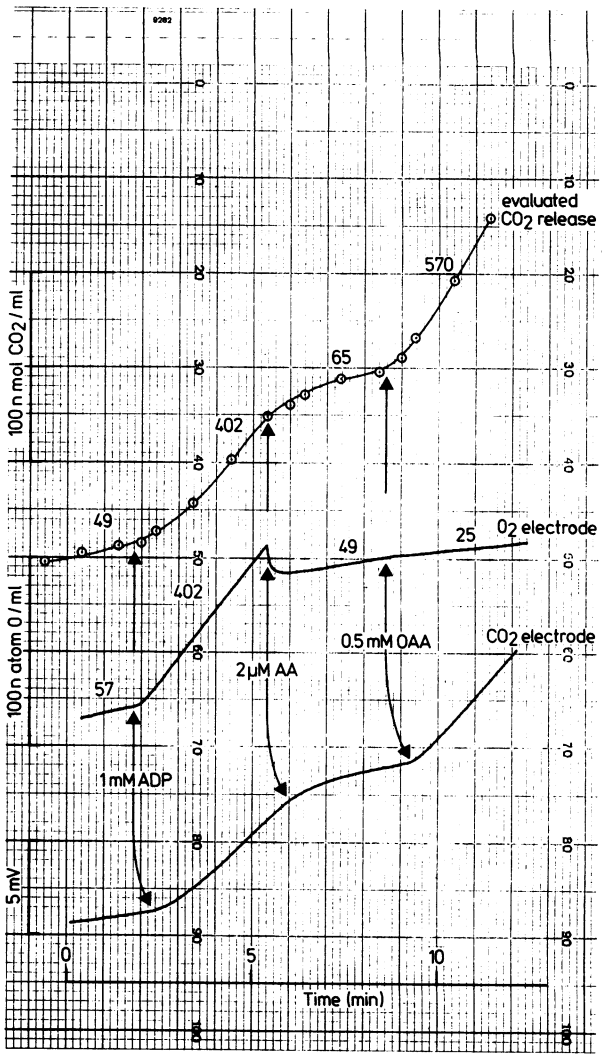

FIG. 7

similar experiment (Fig. 7), the addition of 2 μM AA, an inhibitor of mitochondrial electron transport, inhibited both O_2 uptake and CO_2 release. The further addition of 500 μM OAA then restored CO_2 release to a rate exceeding that of respiration after the initial addition of ADP. Similar results were also obtained with mitochondria from pea leaves (Table I). The CO_2/O_2 quotient during active respiration was 1.0, and the rate of CO_2 release in the presence of OAA largely exceeded the rate of CO_2 release during active respiration. The increased rate of CO_2 evolution determined after the addition of OAA cannot be explained by a decarboxylation of the added OAA, since this had been corrected for (see above). Although the experiments were performed at pH 7.4, at which mitochondria show only low malic enzyme activity (15), the possibility existed that the extra CO_2 released after OAA addition derived from an oxidative decarboxylation of malate which had been formed from OAA. We checked this. In an experiment with spinach mitochondria carried out in analogy to the experiment of Figure 6, in which the rate of CO_2 evolution from glycine in state 3 was 460 and that with glycine plus OAA 900 μmol mg^{-1} Chl h^{-1}, the rate of CO_2 evolution with glycine plus malate (3–10 mM) was 570 μmol mg^{-1} Chl h^{-1}. Thus only a minor part of the extra CO_2 released upon the addition of OAA to mitochondria oxidizing glycine could be accounted for to malate decarboxylation. One can therefore conclude that the increase of CO_2 evolution caused by the addition of OAA is primarily due to an increased rate of glycine decarboxylation.

Table I. *Oxygen Uptake and CO_2 Release by Pea Leaf Mitochondria*

Substrate: 20 mM glycine. The data are the mean of three preparations ± SD.

	Rate	CO_2/O_2
	natom O mg^{-1} prot min^{-1}	*ratio*
Oxygen uptake		
State 3 (0.2 mM ADP)	342 ± 32	
State 4	48 ± 16	
CO_2 release		
1 mM ADP	345 ± 29	1.0
Plus 500 μM OAA	456 ± 62	

It has been generally observed with plant mitochondria that the respiration of NADH linked substrates, *e.g.* glycine, is inhibited by the addition of OAA (3, 6, 13, 16, 17). This is due to a withdrawal of redox equivalents from electron transport by OAA, which enters the mitochondrial matrix and is reduced to malate by intramitochondrial NADH and malate dehydrogenase. The transport of OAA is enabled by a specific translocator with a very high affinity for its substrate (K_m 3–7 μM) and a high maximal activity (7). A malate-OAA exchange, facilitated by this transport, appears to play a role in photorespiration. In the photorespiratory cycle, two molecules of glycine that are oxidized in the mitochondria yield one NADH and one serine, and the

latter, after transamination to hydroxypyruvate, is reduced in the peroxisomes to glycerate. Alternatively redox equivalents required in the peroxisomes can be provided at least in part by the chloroplasts via a malate-OAA shuttle facilitated by another specific OAA translocator in the chloroplast envelope (11). In order to maintain the redox balance of the cell, the proportion of reducing equivalents provided by the chloroplasts may vary according to the metabolic conditions. In situations where the chloroplasts do not contribute, all the NADH formed during glycine oxidation in the mitochondria would be siphoned off by the peroxisomal reduction of hydroxypyruvate and would be therefore no longer available for mitochondrial electron transport. This results from the equilibrium for hydroxypyruvate reduction being far on the side of glycerate, and from the very high activity of the OAA translocator. In a situation where chloroplasts also contribute redox equivalents for hydroxypyruvate reduction, the equivalent amount of redox equivalents generated by glycine oxidation would remain available for mitochondrial electron transport generating ATP. Recent experiments have indicated that mitochondrial ATP synthesis has an important function in the light metabolism of a green plant cell (8).

During normal photosynthesis the mitochondria of a leaf cell must oxidize glycine continuously at high rates. In a spinach leaf, carrying out photosynthesis in air at a rate equal to a net fixation of 200 μmol $CO_2 \cdot mg^{-1}$ Chl$\cdot h^{-1}$, the average rate of ribulosebis-P oxygenation can be estimated as 55 μmol$\cdot mg^{-1}$Chl$\cdot h^{-1}$ (8), which implies a rate of glycine oxidation equal to 27.5 μmol NADH formed$\cdot mg^{-1}$Chl$\cdot h^{-1}$. Using citrate synthase as a mitochondrial marker enzyme, these spinach leaves were found to contain 1.5 mg mitochondrial protein$\cdot mg^{-1}$Chl (H Ebbighausen, unpublished data). From this, the required rate of glycine oxidation for this rate of photorespiration can be calculated as 300 nmol$\cdot mg^{-1}$protein$\cdot min^{-1}$. This rather high value may be regarded as a minimum estimate. To meet the demands of above average rates of photorespiration, which, according to the metabolic situation, may occur, considerably higher rates of glycine oxidation may be required at certain times.

The question therefore arises as to whether the capacity of mitochondrial glycine oxidation is sufficient, particularly in relation to mitochondrial electron transport. In the past a number of investigators have determined rates of glycine oxidation, either by electrode measurement of NH_4^+ release or by radioactive assay of the $^{14}CO_2$ released from [^{14}C]glycine, and have compared these with rates of respiration. In some cases, the addition of OAA to mitochondria, actively respiring with glycine as substrate, resulted in an increase of glycine oxidation (1, 2, 14, 17), whereas in other experiments only small changes in the glycine oxidation rates were observed (3). In general, the observed rates of glycine oxidation were comparatively low and on the basis of these data it has been even questioned whether glycine decarboxylation is the only source of CO_2 release during photorespiration (3).

Our results show that under saturating conditions the rate of glycine oxidation by intact mitochondria can exceed the rate of glycine-dependent respiration, the maximally obtained rates being about twice the minimum values required for the assumed average rates of photorespiration. The decreased glycine oxidation during respiration does not seem to be caused by a limitation of the respiratory chain *per se*, as the respiration with glycine can be increased when another substrate, *e.g.* malate, is added on top of the glycine (our unpublished experiments with mitochondria from pea and spinach). More likely it reflects a redox control of glycine oxidation. The glycine decarboxylase multienzyme complex was recently shown to be inhibited by NADH (5). Such an inhibition explains that in mitochondria oxidizing glycine the RCR can be exceptionably high (RCR = 15.5 in Fig. 5, RCR = 19 in Ref. 7), much higher than with other substrates (7). Therefore, the increase of glycine oxidation upon the addition of OAA, shown in the preceding, may be the result of a lowering of the mitochondrial NADH level by OAA reduction.

In summary, our data show that the rate of mitochondrial glycine oxidation can considerably exceed the respiratory capacity of the mitochondria with glycine as substrate, if the NADH generated in the mitochondria is withdrawn by a malate-OAA shuttle.

LITERATURE CITED

1. ARRON GP, MM SPALDING, GE EDWARDS 1979 Stoichiometry of carbon dioxide release and oxygen uptake during glycine oxidation in mitochondria isolated from spinach. Biochem J 184: 457–460
2. BERGMAN A, J ERICSON 1983 Effect of pH, NADH, succinate and malate on the oxidation of glycine in spinach leaf mitochondria. Physiol Plant 59: 421–427
3. DAY DA, JT WISKICH 1981 Glycine metabolism and oxaloacetate transport by pea leaf mitochondria. Plant Physiol 68: 425–429
4. DELIEU T, WALKER DA 1972 An improved cathode for the measurement of photosynthetic evolution by isolated chloroplasts. New Phytol 71: 201–225
5. DOUCE R 1987 Organisation of the Respiratory Chain. *In* AL Moore, RB Beechey, eds, Plant Mitochondria: Structural, Functional and Physiological Aspects. Plenum Press, New York. In press
6. DOUCE R, WD BONNER 1972 Oxaloacetate control of Krebs cycle oxidation in purified plant mitochondria. Biochem Biophys Res Commun 47: 619–624
7. EBBIGHAUSEN H, J CHEN, HW HELDT 1985 Oxaloacetate translocator in plant mitochondria. Biochim Biophys Acta 810: 184–199
8. EBBIGHAUSEN H, MD HATCH, RMcC LILLEY, S KRÖMER, M STITT, HW HELDT 1987 On the functions of malate-oxalacetate shuttles in a plant cell. *In* AL Moore, RB Beechey, eds, Plant Mitochondria: Structural, Functional and Physiological Aspects. Plenum Press, New York. In press
9. FARQUHAR GD, S VON CAEMMERER 1982 Modelling of photosynthetic response to environmental conditions. *In* OL Lange, PS Nobel, CB Osmond, H Ziegler, eds, Encyclopedia of Plant Physiology (New Series), Vol 12b. Springer-Verlag, Berlin, pp 549–587
10. HATCH MD, HW HELDT 1985 Synthesis, storage and stability of [4-^{14}C] oxaloacetic acid. Anal Biochem 145: 393–397
11. HATCH MD, L DRÖSCHER, UI FLÜGGE, HW HELDT 1984 A specific translocator for oxaloacetate transport in chloroplasts. FEBS Lett 178: 15–19
12. HÜTTER JF, HM PIPER, PG SPIECKER 1983 A new method for continuous measuring of respiratory quotient in a computer-assisted working heart preparation. Basic Res Cardiol 78: 1–7
13. JOURNET EP, M NEUBURGER, R DOUCE 1981 The role of glutamate-oxaloacetate transaminase and malate dehydrogenase in the regeneration of NAD^+ for glycine oxidation by spinach leaf mitochondria. Plant Physiol 67: 467–469
14. MOORE, AL, C JACKSON, B HALLIWELL, JE DENCH, DO HALL 1977 Intramitochondrial localization of glycine decarboxylase in spinach leaves. Biochem Biophys Res Commun 78: 483–491
15. TOBIN A, B DJERDJOUR, E JOURNET, M NEUBURGER, R DOUCE 1980 Effect of NAD on malate oxidation in intact plant mitochondria. Plant Physiol 66: 225–229
16. WALKER DA, H BEEVERS 1956 Some requirements for pyruvate oxidation by plant mitochondrial preparations. Biochem J 62: 120–127
17. WOO KC, CB OSMOND 1976 Glycine decarboxylation in mitochondria isolated from spinach leaves. Aust J Plant Physiol 3: 771–785