Review Letter

PHOTORESPIRATION – STILL UNAVOIDABLE?

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1. Introduction

Photorespiration, a curious apparent reversal of the photosynthetic carbon metabolism of plants, results in the light-dependent uptake of O_2 and release of CO_2 [1–8]. In some circumstances this partial reversal severely reduces the photosynthetic potential of C_3 plants, with estimates of this reduction ranging as high as 50% for some plants of economic importance [1–8]. Although considerable knowledge about the biochemical mechanisms of photorespiration has accumulated, we still seem no nearer to an understanding of the reasons for this apparently counterproductive metabolic behaviour.

Recently a considerable body of evidence has been amassed [4–6] which strongly suggests that the first step of the photorespiratory pathway is the synthesis of 2-phosphoglycolate(P-glycolate) via the oxygenolytic cleavage of ribulose-1,5-bisphosphate (RuP_2) catalysed by the same enzyme that catalyses the initial fixation of CO_2, now termed RuP_2 carboxylase oxygenase (EC 4.1.1.39) [9–12]. P-glycolate is then further metabolized by the photorespiratory carbon oxidation (PCO) or glycolate pathway to yield the CO_2 that is released during photorespiration [5,12,13]. One formulation of the PCO pathway, showing it as a cycle interlocked with the photosynthetic carbon reduction (PCR) cycle, is given in fig.1. Two molecules of P-glycolate produced by the RuP_2 oxygenase reaction are converted by this cycle to a molecule of CO_2 and a molecule of 3-phosphoglycerate (P-glycerate), the latter re-entering the PCR cycle. This cycle concept of the PCO pathway will be discussed later.

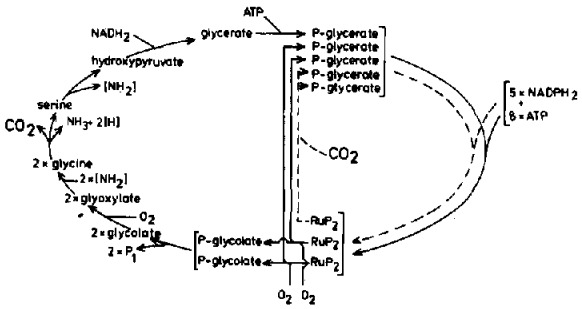

Fig.1. Photorespiratory carbon oxidation (PCO) cycle (solid lines) and photosynthetic carbon reduction (PCR) cycle (dashed lines) interlocked at the CO_2 compensation point.

In 1973 we suggested that the existence of photorespiration could be understood if the mechanism of carboxylation of RuP_2 obligatorily involves an enzyme-bound intermediate which is capable of reacting with O_2 as well as with CO_2 [14]. It follows that RuP_2 oxygenation and, therefore, photorespiration is the unavoidable consequence of the active site chemistry of RuP_2 carboxylase-oxygenase and the relative concentrations of CO_2 and O_2 at the active site. This hypothesis is based upon several

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assumptions and lends itself to several predictions. In 1973 there was little evidence concerning these assumptions and predictions. Our purpose in this communication is to consider their validity in the light of evidence which has since accrued.

The assumptions and predictions that we wish to examine are as follows:

1. RuP_2 oxygenase is the major mechanism for the synthesis of glycolate.

2. Carboxylation and oxygenation of RuP_2 occur at the same catalytic site.

3. The ratio of carboxylation to oxygenation is determined by the relative concentrations of CO_2 and O_2.

4. All RuP_2 carboxylases, regardless of taxonomic origin, should also exhibit RuP_2 oxygenase activity

5. The PCO pathway should act as a carbon scavenger and therefore should be cyclic.

We also discuss the status of photorespiration in plants lacking its external manifestations and attempt to assess the necessity or otherwise of photorespiration to plant metabolism. We conclude that the hypothesis that photorespiration is unavoidable is still tenable.

2. Discussion

2.1. *Is RuP_2 oxygenase the major mechanism for the synthesis of glycolate?*

In a qualitative sense it would be dangerous to claim that RuP_2 oxygenase, coupled to P-glycolate phosphatase, is the sole mechanism for glycolate synthesis. Quantitative evidence, however, indicates that this mechanism is responsible for most of the glycolate synthesized.

In vitro ^{18}O studies have shown that the RuP_2 oxygenase reaction involves the incorporation of one atom from molecular oxygen into the carboxyl group of P-glycolate [11]. A simple precursor–product relationship therefore exists between oxygen and glycolate which is open to direct in vivo experimental verification for clearly, if the oxygenase reaction is the major pathway of glycolate synthesis, the enrichment of ^{18}O in the carboxyl group of glycolate should equal or approach that of ^{18}O in the O_2. Analyses of a number of photosynthetic systems capable of forming glycolate (*Chromatium, Chlorella, Euglena* and intact spinach chloroplasts) have established that at least 90% of the glycolate is synthesized by a pathway which brings about the incorporation of one atom from O_2 [15–17]. Even with intact leaves where the enrichment of the isotope within the leaf cannot be precisely measured owing to the dilution of the isotope with photosynthetically-produced O_2, a comparison of the specific activities of glycolate and the O_2 outside the leaf – a comparison which gives a minimum estimate – shows that at least 60–80% of the glycolate is synthesized in the same manner [18–20]. While this does not prove that the RuP_2 oxygenase reaction is responsible for the incorporation of the isotopic oxygen in vivo – other oxygen incorporating reactions could conceivably contribute – it does set considerable constraints upon the types of mechanism which can be accommodated.

Inhibitor studies with intact isolated chloroplasts also support the above assumption [21,22]. Glycolate synthesis from triose or pentose monophosphates by isolated chloroplasts is almost completely (> 90%) dependent upon photophosphorylation. Since the ribulose-5-P kinase reaction leading to the formation of RuP_2 is the only relevant reaction requiring ATP, this result strongly suggests that RuP_2 is the major (> 90%) source of glycolate. When intact isolated chloroplasts, fixing $^{14}CO_2$, were treated with fluoride, an inhibitor of P-glycolate phosphatase, label accumulated in P-glycolate rather than in glycolate [23]. This result is in accord with the view that P-glycolate, the product of the RuP_2 oxygenase reaction, is the precursor of glycolate.

The RuP_2 oxygenase mechanism attracted some early criticism on the grounds that there did not seem to be sufficient activity in vitro to account for the known rates of glycolate synthesis and photorespiration in vivo [3,24]. The same criticism could also have been made of the RuP_2 carboxylase activity vis a vis the in vivo rate of photosynthesis although few doubted that the carboxylation of RuP_2 represents the first step in the photosynthetic fixation of CO_2. Failure to activate the enzyme before assay lead to spuriously high values for the K_m (CO_2) of the carboxylase.

Similarly, incompletely activated enzyme and CO_2 contamination of the reaction mixtures are the most probable causes for the low rates of RuP_2 oxygenase then recorded. With the development of improved procedures [25,26], rates of RuP_2 oxygenase in the order of 80–100 μmol/mg chlorophyll.h at 25°C have been recorded [25–27]. These rates are quite adequate to account for the known in vivo rates of glycolate synthesis and photorespiration [28,29].

Thus the rate of the RuP_2 oxygenase reaction is adequate. It, and no other mechanism so far proposed, incorporates an atom of oxygen from O_2 as is required by the in vivo ^{18}O labelling evidence and its kinetic parameters are consistent with all the known characteristics of photorespiration. An affirmative answer to the above question seems justified.

2.2. *Do carboxylation and oxygenation of RuP_2 occur at the same catalytic site?*

All present evidence favours an affirmative answer to this question also. Both catalytic activities reside on the larger of the enzyme's two types of subunits [30,62]. CO_2 and O_2 compete with one another in a manner that is classically, linearly competitive [27,28]. This kinetic behaviour is consistent with CO_2 and O_2 competing with each other for the same enzyme-bound intermediate, as we originally proposed [14], or, and possibly more likely, for different intermediates in rapid equilibrium with each other.

Recent studies of the activation–inactivation properties of the enzyme showed that carboxylase and oxygenase activities varied in parallel during these transitions [25,26,31,32] – kinetic responses consistent with a common active site.

A number of compounds are known which are thought to react with the enzyme at the RuP_2 binding site. Among these are the carboxylase transition state analogue, carboxyribitol-1,5-bisphosphate [33–35], the substrate analogue xylitol-1,5-bisphosphate [36] and the affinity labels *N*-bromoacetylethanolamine phosphate [37] and pyridoxal-5′-phosphate [38]. The inhibition or inactivation elicited by these reagents is the same for the carboxylase as it is for the oxygenase.

2.3. *Is the ratio of carboxylation to oxygenation determined by the relative concentrations of CO_2 and O_2 at the active site?*

Early evidence seemed strongly contrary to this prediction. The ratio of activities, carboxylase to oxygenase, seemed to vary during the purification of the enzyme and during subsequent storage (see table 1 and fig.5 in [10]). The activity ratios reported for different species were widely different and were even reported to respond to fertilizer treatment [39]. The pH-activity profiles of the two reactions seemed remarkably different [10] and various sugar phosphates were reported to elicit differential effects upon the two reactions [35]. However, the assay procedures employed during these experiments inadvertently overlooked a most important phenomenon, the relatively slow activation of the enzyme by CO_2 and Mg^{2+} first described in [40]. In the absence of CO_2 and Mg^{2+}, the enzyme becomes inactive but may be reactivated by restoration of the CO_2 and Mg^{2+} [31]. Carboxylase and oxygenase activities respond identically [26,31]. These transformations are not instantaneous and are equilibrium processes with the degree of activation at equilibrium depending on pH and on the concentrations of CO_2 and Mg^{2+} [31]. Thus, in the early studies, the kinetics of catalysis were obscured by the kinetics and equilibria of activation. Even in those studies where pre-activation was attempted, the previously universal practice of using the same CO_2 concentration during both pre-activation and assay gave rise to spuriously high K_m (CO_2) values and sigmoidal kinetic behaviour [41,42,103]. These studies also showed that the assay of RuP_2 oxygenase needs to be approached with considerable caution. While quite high concentrations of CO_2 are required during preincubation to ensure full activity, the presence of quite low CO_2 concentrations during assay cause competitive inhibition of the oxygenase activity [25].

When precautions were taken to ensure that the activation state of the enzyme was the same for both carboxylase and oxygenase assays and CO_2 contamination of the oxygenase assay solutions was minimized, all of the apparent inconsistencies referred to above disappeared. The activity ratio remained constant throughout purification and subsequent storage of the enzyme (G.H.L., unpublished). The differential effects of various sugar phosphates were shown to be artifacts [32]. The pH-activity profiles for the carboxylase and the oxygenase reactions were then found to be quite similar, although not quite identical [43,44]. Indeed it is possible that, if the last traces of CO_2 contamination could be removed completely from the

oxygenase assay solutions, even this small difference might disappear. In this respect it is relevant that the carboxylase assays were performed at saturating CO_2 concentrations over the complete pH range and thus represent essentially V_{max} values. However, the oxygenase assays were conducted with solutions in equilibrium with CO_2-free air (i.e., 250 μM O_2), a sub-saturating concentration. At sub-saturating substrate concentrations the influence of a competitive inhibitor, such as CO_2 in this case, becomes manifest. As the pH increases, the influence of a given quantity of bicarbonate contamination decreases since the concentration of CO_2, the species responsible for inhibition, declines. The net effect is that the pH profile for oxygenase activity, as determined with sub-saturating O_2 concentrations, is shifted to more alkaline pH values.

Data concerning the carboxylase to oxygenase activity ratio of enzyme from a broad spectrum of plants is lacking because the essential precautions referred to above have not often been observed. A limited survey of C_3 plants only (Badger and G.H.L., unpublished) revealed very little difference in this ratio.

Several reports [45–47] of genetic differences in kinetic properties of RuP_2 carboxylase-oxygenase cannot be adequately assessed, once again because the requisite precautions in activation and assay techniques were not taken. In one report where the precautions were adequate, the small difference between K_m (CO_2) values for the carboxylases from *Panicum milioides*, the putative C_3–C_4 intermediate species, (17 μM) and soybean (25 μM) was 'less than could be reliably established by standard assay techniques' [48].

A report [49] that glycidate (2,3-epoxypropionate) brings about the differential inhibition of the oxygenase activity has not been substantiated, despite the efforts of at least three independent groups (personal communications: Paech and Tolbert, Michigan State University; Chollet, University of Nebraska; G.H.L., unpublished).

2.4. *Do all RuP_2 carboxylases, regardless of their taxonomic origin, possess RuP_2 oxygenase activity?*

It is a consequence of the implication present in our hypothesis that only one chemistry for the carboxylation of RuP_2 is available to living organisms under present or past biospheric conditions. Certainly only one mechanism appears to have evolved. Oxygenase activity has been detected in RuP_2 carboxylase preparations wherever it has been sought, regardless of the taxonomic origin of the enzyme. Thus oxygenase activity has been demonstrated in RuP_2 carboxylase preparations from such taxonomically diverse sources as the chemosynthetic bacteria *Alcaligenes eutrophus* [50,51] and *Thiobacillus intermedius* [51], the photosynthetic bacteria *Chromatium vinosum* [16,52], *Rhodospirillum rubrum* [53,54] and *Ectothiorhodospira* [51], the blue–green alga *Alphanocapsa* 6308 [55], the green algae *Chlamydomonas* [45,56] Euglena [57], *Halimeda* [58] and *Chlorella* [59] and diverse angiosperms with C_3, C_4 and Crassulacean acid metabolism ([60,61], Badger and G.H.L., unpublished).

A more rigorous survey embracing the complete taxonomic spectrum of organisms in which RuP_2 carboxylase-oxygenase is found and one which determined not only the ratio V_{max} carboxylase to V_{max} oxygenase but also the ratio K_m (CO_2) to K_m (O_2) would be most instructive in determining whether or not these ratios have remained constant throughout the course of evolution. Although the enzyme from the photosynthetic bacterium, *Chromatium*, appears to be activated in much the same manner as the spinach leaf enzyme [16], there is no guarantee that the application of activation and assay techniques found suitable for the enzyme from eucaryotes will necessarily be appropriate for the enzyme from procaryotes. For example, the ability to detect oxygenase activity depends to some extent on the relative slowness with which the activated enzyme collapses back to the inactive form in the 'CO_2-free' conditions of the oxygenase assay [25]. If a carboxylase did exist whose rate of inactivation was very much more rapid, one might be unable to detect oxygenase activity using the standard procedures.

2.5. *Does the PCO pathway act as a carbon scavenger?*

Within the constraints of the biochemistry available to them, organisms should conserve reduced carbon rather than squander it. One would therefore expect that as much of the P-glycolate carbon as possible would be re-cycled back to the PCR cycle as P-glycerate as shown in fig.1 and not, as proposed [3,24], be mostly oxidized to CO_2. This cyclic formulation of the PCO pathway, with the decarboxylation of glycine

as the sole source of photorespiratory CO_2, has been criticized [3,24] on two grounds.

1. The rates of glycine decarboxylation by isolated leaf mitochondria were previously insufficient to sustain the known in vivo rates of photorespiration.

However, with improved techniques [76–79] the rates of glycine decarboxylation by isolated mitochondria now appear to be quite adequate.

2. This formulation of the PCO pathway allows a loss of only 25% of the carbon in glycolate as CO_2 whereas, according to [3,24], photorespiratory CO_2 loss in air often exceeds 50% of net CO_2 fixation during photosynthesis.

This criticism fails to acknowledge the cyclic nature of the process and implies that a rigid stoichiometry exists between the PCO and PCR pathways. Such a one-to-one stoichiometry does indeed exist at the CO_2 compensation point where every carbon fixed by the PCR cycle is balanced by the loss of one by the PCO cycle (fig.1). Thus the cyclic integrated formulation of the PCO and PCR pathways permits the loss of 100% and more of the carbon fixed by the PCR cycle, not merely 25%. At CO_2 concentrations above the CO_2 compensation point the PCR cycle (expressed as the rate of CO_2 fixed) turns faster than the PCO cycle (expressed as the rate of CO_2 evolved). The result is a net carbon gain for the plant. Below the CO_2 compensation point the PCR cycle turns more slowly than the PCO cycle and more than 100% of the CO_2 fixed by the PCR cycle is released, the excess being supplied from the plant's reserves of reduced carbon.

That the PCO pathway is a cycle implies that mass flow occurs from P-glycolate to P-glycerate. Experimental evidence for this was recently obtained by ^{18}O-labelling studies [5,20]. The isotope, supplied as O_2, was shown to flow sequentially round the cycle from glycolate through glycine and serine to P-glycerate. Considerable dilution of the isotope in P-glycerate was evident as was to be expected since unlabelled P-glycerate is formed directly from RuP_2 by both carboxylation and oxygenation. At the CO_2 compensation point the dilution of the isotopic oxygen in P-glycerate was experimentally determined [20]. It approached the value of 20% that predicted from the balanced integrated formulation of the PCO and PCR cycles at the CO_2 compensation point (fig.1), with 75% of the carbon entering the PCO cycle as P-glycolate being recovered as P-glycerate. The ^{18}O in the carboxyl group of P-glycerate cannot be recycled to RuP_2 (in the same manner as ^{14}C for example), and thus further accumulate in the PCO cycle intermediates, because it is exchanged with the medium when the carboxyl group is reduced. The previous failure to observe isotopic oxygen in P-glycerate [18] can be attributed to the use of insufficiently enriched $^{18}O_2$ to allow for this dilution.

The experimentally determined rates of turnover of glycine and serine in vivo [1,63–68] are also in accord with a cyclic formulation of the PCO pathway. These rates are sufficient to account for photorespiration with the stoichiometry of one carbon atom released as CO_2 for every 4 carbons in flux. In air the total flux of carbon through the PCO cycle was measured to be 90% of the true rate of photosynthesis [64]. If all this carbon was lost as CO_2, the differences in the quantum yields of photosynthesis in air as opposed to 2% O_2 would be considerably larger than those measured [69].

3. RuP_2 carboxylase-oxygenase in plants lacking the external manifestations of photorespiration

Three classes of plants are known to lack the external symptoms of photorespiration, the most notable of these symptoms being the inhibition of photosynthesis by O_2, the existence of a high CO_2 compensation point (30–50 ppm CO_2 when measured at 25°C with 21% O_2) and the sensitivity of the CO_2 compensation point to O_2. These classes are:

(i) C_4 plants.
(ii) Plants with Crassulacean acid metabolism (CAM).
(iii) Many algae when grown on limiting concentrations of inorganic carbon.

Since the kinetic properties of the RuP_2 carboxylase-oxygenase enzymes from these classes of plants are quite similar to those of the enzymes from plants which do manifest photorespiration, other explanations for this suppression of photorespiration must be sought. One possibility is that the apparently photorespiration-less plants have a mechanism which raises the CO_2 concentration at the site of the enzyme to levels considerably above those achieved by equilibration with atmospheric CO_2 levels. This would permit carboxylation to compete more effectively with oxy-

genation for enzyme-bound RuP_2 and thus stimulate CO_2 fixation and suppress photorespiration.

In C_4 plants anatomical differentiation plays an important role. Salient features are the sequestration of RuP_2 carboxylase-oxygenase in the chloroplasts of the bundle sheath cells where it is inaccessible to external CO_2 and the transport of CO_2 in the form of β-carboxyl groups of C_4 acids from the mesophyll cells where it is first fixed to the bundle sheath cells where it is released and refixed by the PCR cycle [70,71]. It has been suggested that the decarboxylation of the C_4 acids within the bundle sheath cells gives rise to a CO_2 concentration considerably in excess of the concentration of CO_2 in air-equilibrated solution [9,14]. The demonstration of an intermediate internal pool of CO_2 in C_4 leaves [73] supports this suggestion. Thus, although photosynthesis by bundle sheath cells isolated from C_4 plants is similar to C_3 photosynthesis in its sensitivity to oxygen inhibition [74], this sensitivity is not apparent in vivo because of the high CO_2 concentration prevailing at the site of RuP_2 carboxylase-oxygenase.

In CAM plants the separation of C_4 acid formation from C_4 acid decarboxylation is achieved by temporal rather than spatial means as in C_4 plants [72]. Since decarboxylation in CAM plants occurs at a time when the stomata are closed, the internal concentration of CO_2 may well rise considerably above ambient thus achieving the same stimulation of carboxylation at the expense of oxygenation, and hence of photorespiration, that is suggested for C_4 plants. The ability of O_2 to inhibit the photosynthesis of the CAM plant *Kalanchoe diagremontiana* was shown to be dependent upon suppression of the CAM mechanism by manipulation of the growth conditions [75]. This result is again consistent with the explanation for the lack of photorespiration offered above.

The case of some algae adapted to limiting levels of inorganic carbon is very different for here there are no gross anatomical features to facilitate an elevation of the CO_2 concentration, as in the C_4 plants. Yet these algae fail to show the symptoms of photorespiration (O_2 inhibition of photosynthesis, glycolate excretion) that are readily apparent when the same algae are cultured under CO_2 enriched conditions [56,80–85]. It has been suggested [56] that the cells adapted to growth on limiting carbon may have a CO_2 concentrating mechanism. This may be induced during adaptation to the carbon limited conditions along with a similar induction of carbonic anhydrase [84,86–90] which may be required to catalyse one step of the mechanism. An active uptake of bicarbonate ions by algal cells adapted to limiting CO_2 concentrations has been reported [91–94]. However, whether or not this active bicarbonate uptake system also leads to an increase in the internal concentration of CO_2, as is required if the oxygenation of RuP_2 is to be suppressed, would depend on the internal pH. An increase in the internal concentration of bicarbonate over that outside, purely as a result of internal alkalization, as appears to happen with isolated intact chloroplasts [95], would not achieve the necessary increase in the CO_2 concentration. Concurrent measurements of the internal and external pH and bicarbonate concentrations are needed to conclusively prove or disprove the existence of a CO_2 concentrating mechanism in these algae.

4. Is photorespiration essential or merely unavoidable?

Photorespiration has persisted in C_3 plants throughout evolution. This suggests either that photorespiration serves a vital function which remains to be determined or that it serves no useful function but has persisted owing to the inseparable nature of the carboxylation and oxygenation reactions. If photorespiration does indeed fulfill a vital function, regardless of what that might be, one might well question the wisdom of attempting to suppress it by genetic or chemical means. The existence of plants (C_4 and CAM) in which photorespiration is severely suppressed and the fact that the vegetative growth of C_3 plants can be accelerated either by raising the CO_2 concentration or by lowering the O_2 concentration [100,101] suggests that photorespiration is not essential for the plant's well being.

RuP_2 oxygenase aside, is the PCO pathway necessary for any purpose other than the retrieval of the carbon lost from the PCR cycle as P-glycolate? Since the cell's requirement for glycine and serine would be satisfied by a very small fraction of the flux into the PCO pathway, the production of these amino acids cannot be the principal function of photorespiration. Alternatively, it has been suggested [96–99] that the function of photorespiration is to assist in the degrada-

tion of excess photochemically-generated energy. In some circumstances, such as, for example, when the leaf's stomata are closed in the light due to water stress, it is argued that a potentially deleterious build-up of reducing equivalents might occur unless a means of 'short circuiting' photosynthesis is available. Photorespiration certainly does consume energy. Indeed, at the CO_2 compensation point, this is the only net result of the integrated PCO and PCR cycles. But it does not logically follow that photorespiration is therefore necessary for this purpose. It is certainly not unique in its energy dissipating function. Rather, we take the view that photorespiration and the energy dissipation which accompanies it are the consequences of the RuP_2 oxygenase reaction which occurs unavoidably. Of course, the two extremes of this essentially versus unavoidability debate are not necessarily mutually exclusive. Photorespiration would certainly assist in energy dissipation, in concert with other reactions, if and when overproduction occurred. However, the presence of RuP_2 oxygenase activity in photosynthetic anaerobes [16,51–54] and in non-photosynthetic organisms [50,51,102] argues against RuP_2 oxygenase and photorespiration being specific adaptations for the dissipation of excess photochemically generated energy.

Evidence which has accumulated since we first proposed that photorespiration is the inevitable consequence of the active site chemistry of RuP_2 carboxylase-oxygenase, and the present atmospheric concentrations of CO_2 and O_2, has tended to substantiate and extend our hypothesis rather than disprove it. However, the hypothesis cannot be established or otherwise until further information about the mechanism of this unique enzyme from the full range of organisms in which it occurs is obtained or until an oxygenase-less RuP_2 carboxylase is discovered.

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