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Autotrophic CO_2 Assimilation and the Evolution of Ribulose Diphosphate Carboxylase

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INTRODUCTION

Although the carbon-fixing paths reflect but one aspect of the evolution of autotrophs, the similarity of these paths in all autotrophic organisms promises insights about evolution not yet available from an examination of the diverse exergonic processes with which CO_2 fixation is coupled. Accordingly, a comparison of these pathways and catalysis and regulation within them is undertaken in this article.

Although the primary focus is on autotrophic paths of CO_2 fixation, the assimilation of more reduced one-carbon compounds is also briefly considered in an evolutionary context. No consideration, however, is given to energy-trapping mechanisms or reverse electron flow (38) in autotrophs. For a more comprehensive treatment of autotrophy in prokaryotes, including its basis, the review of D. P. Kelly is excellent (112), as is the earlier review of the photosynthetic bacteria by Pfennig (167). In composing the present review, references to meeting abstracts or personal communications have generally been avoided. Rare exceptions involve experimental work with which I am familiar.

For the sake of precision, the following nomenclature is used to describe microorganisms on the basis of energy sources utilized (132). Photolithotrophy and photoorganotrophy refer to the capacity for light-dependent growth requiring exogenous, inorganic hydrogen donors

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and organic hydrogen donors, respectively. Chemolithotrophy and chemoorganotrophy refer to the capacity for growth in the dark that is dependent upon the oxidation of exogenous, inorganic substances and organic substances, respectively.

AUTOTROPHIC CO_2 ASSIMILATION

Pathways in Plant Photolithotrophs

Chemical similarities between photosynthesis in bacteria and green plants were first recognized in 1930 by C. B. Van Niel and later expanded and summarized by him (233). Basically he postulated that the two processes were examples of photochemical reduction of carbon dioxide with different hydrogen donors, and from this he derived the well-known general equation for photosynthesis: $CO_2 + 2H_2A \xrightarrow{light} (CH_2O) + H_2O + 2A$. In the case of green plants, H_2A was H_2O and 2A was therefore O_2, whereas in bacteria 2A was not O_2 and H_2A was variable. The demonstration with illuminated chloroplasts in 1937 by Hill (85) that ferric ion could replace CO_2 as an oxidant with resultant O_2 evolution from water separated the reduction of CO_2 from light-dependent O_2 formation. Thus increased attention was focused upon the possibility that the reduction of CO_2 was a dark reaction.

There followed at Berkeley the elegant elucidation of the chemistry of the dark reactions in plants that requires no detailed reiteration. In essence, Calvin and his colleagues proved that the reductive pentose phosphate cycle is the main CO_2 fixation path in all green plants examined (for a review, see 33). The first stable product of CO_2 fixation was 3-phospho-D-glycerate which was derived from the carboxylation of D-ribulose-1,5-diphosphate (RuDP) catalyzed by RuDP carboxylase. Other enzymes that ostensibly also functioned uniquely in plant photolithotrophic CO_2 assimilation were phosphoribulokinase, D-sedoheptulose-1,7-diphosphatase, alkaline D-fructose-1,6-diphosphatase, and nicotinamide adenine dinucleotide phosphate-linked D-glyceraldehyde-3-phosphate dehydrogenase. Among plants the widespread distribution of these and other catalysts in the reductive pentose phosphate (Calvin) cycle left little doubt about the ubiquity of the cycle and implied its function in these species.

It was therefore of considerable interest when Hatch and Slack (76), extending the earlier observations of Kortshak et al. (120), provided evidence for the function of a new carboxylation pathway in sugar cane leaves. Their data supported rapid incorporation of $^{14}CO_2$ into C-4 of malate and aspartate and a slower incorporation into C-1 of 3-phosphoglycerate. Similar observations were made with other *Gramineae* (79) and in species from the families *Cyperaceae*, *Amaranthaceae* (79, 100), *Portulacaceae* (77), and *Chenopodiaceae* (100, 161). Of particular significance, however, were subsequent experiments in which the rate of loss of radioactivity from the dicarboxylic acids was shown to equal the rate of labeling of 3-phosphoglycerate in maize leaves (101). Because of these and related observations and reports that RuDP carboxylase was present at unusually low levels in several of these angiosperms (100, 201), Hatch and Slack postulated a transcarboxylation reaction in which C-4 of the dicarboxylic acids derived from CO_2 (or HCO_3^-) was transferred to RuDP to yield two molecules of phosphoglycerate (201). To date, however, a major weakness of this interpretation has been the failure to detect such a transcarboxylation. Moreover, Bjorkman and Gauhl have reported much higher specific activities of RuDP carboxylase in leaves of "C_4-dicarboxylic acid pathway plants" than those previously described (23). In view of this, Hatch and Slack now consider it much more likely that the transfer of carbon from the C-4 of a dicarboxylic acid to the C-1 of a 3-phosphoglycerate proceeds by decarboxylation followed by RuDP carboxylase-catalyzed refixation of the released CO_2 (78). Recent evidence with isolated corn chloroplasts, however, casts doubt on that interpretation. O'Neal et al. have established that corn plastids fix CO_2 at significant rates via the Calvin cycle without the operation of a cycle involving the C-4 compounds, malate and aspartate (158). Whatever the function of the C_4-dicarboxylic acid pathway, it must be emphasized that the initial carboxylation does not in itself account for net CO_2 fixation by plants in which it occurs. Whether it indeed contributes to the concentration of CO_2 for utilization via catalysis by RuDP carboxylase remains to be established, and Walker has assessed some of the evidence bearing on this point (238). In summary, the Calvin cycle is the sole path that accounts for net reductive CO_2 fixation in plants.

Pathways in Photolithotrophic and Photoorganotrophic Bacteria

The reductive carboxylic acid cycle. Among the strictly photolithotrophic bacteria, Sadler and Stanier (193) found that acetate was the most effective organic compound in elevating cell yields of *Chlorobium limicola* growing in the light on reduced sulfur compounds and CO_2. The definitive studies of Hoare and Gibson on

C. thiosulfatophilum (88) and by Hoare on *Rhodospirillum rubrum* (87), a photoorganotroph, first suggested the function of an unusual pathway in the assimilation of acetate. The isotopic data are summarized with respect to glutamate biosynthesis in Table 1. In the case of *C. thiosulfatophilum*, ^{14}C-bicarbonate or ^{14}C-acetate was provided during growth for 24 h. In experiments with *R. rubrum*, washed malate-grown cells suspended in water were exposed in the light to radioisotope for 1 min. In neither organism could function of the tricarboxylic acid cycle account for the biosynthesis of glutamate from acetate, for if it had, label from [2-^{14}C] acetate would have been incorporated first into C-4 but rapidly into C-3 and C-2 of glutamate.

In 1966 Evans et al. (57) proposed a new ferredoxin-dependent carbon reduction cycle in *C. thiosulfatophilum*. This cycle involved several enzymes of the tricarboxylic acid cycle in addition to two new reductive carboxylations catalyzed by pyruvate and α-ketoglutarate synthases, both of which required reduced ferredoxin. One turn of the cycle resulted in the incorporation of four molecules of CO_2. All enzymes of the cycle were apparently present in extracts although the presence of citrate lyase was not unequivocally demonstrated. Glutamate contained 75% of the label after 30-s $^{14}CO_2$ fixation in the light by suspensions of *C. thiosulfatophilum* in growth medium. Presumably the glutamate was derived from α-ketoglutarate, an intermediate in the postulated cycle. Of particular interest was the fact that the labeling pattern of glutamate observed earlier by Hoare and Gibson could be accounted for by the carbon reduction cycle although their data were obtained with cells which had assimilated acetate in the presence of CO_2. Unfortunately, the kinetic experiments of Evans et al. (57) did not include fixation times of less than 30 s. Thus early rates of labeling of glutamate versus 3-phosphoglycerate were not assessed, rendering it impossible to evaluate the relative contributions of the reductive carboxylic acid and Calvin cycles. In earlier experiments on the same species, Smillie et al. (203) had, in fact, reported the presence of all catalysts in the Calvin cycle.

Sirevag and Ormerod (200) more recently identified some products of $^{14}CO_2$ fixation by *C. thiosulfatophilum*. Their interpretation that fixation occurred predominantly via the reductive carboxylic acid cycle was, however, without conclusive support because fixation products were analyzed after 15-min intervals of $^{14}CO_2$ fixation. Recently, Buchanan and colleagues

TABLE 1. *Origin of glutamate carbons from acetate and bicarbonate*

Carbon skeleton	*C. thiosulfatophilum*[a]	*R. rubrum*
CO_2^-	b*	b
$CH\overset{+}{N}H_3$	(c)	c
CH_2	c, m	m
CH_2	c, m	m
CO_2^-	(c)	c

[a] Abbreviations: b, bicarbonate derived (b* was implied to be bicarbonate derived); c and m, derived from the carboxyl and methyl carbons of acetate, respectively. The notation (c) reflects 15 to 30% as much specific radioactivity as c.

extended their studies of CO_2 assimilation by *C. thiosulfatophilum* Tassajara (32). Using cells grown on 40 mM Na_2CO_3 as the sole carbon source, they found that fixation products were profoundly influenced by the molarity of the $^{14}CO_2$, $H^{14}CO_3^-$, plus $^{14}CO_3^{-2}$. At a total concentration of 40 mM, the percentage of CO_2 fixed into glutamate, α-ketoglutarate, and glutamine was 50% at 5 s and was 28% into citrate plus isocitrate in the same interval. Both percentages showed a negative slope when plotted against time, as would be expected were these compounds early intermediates. No incorporation of $^{14}CO_2$ into sugar phosphate + phosphoglycerate was observed in times up to 25 s. In contrast, at a total concentration of CO_2, bicarbonate, plus carbonate of 0.8 mM, which is ninefold higher than that dissolved in H_2O in equilibrium with air at a pH of 7.3 and 25 C (230), phosphoglycerate was the principal labeled product after 5 s. Moreover, the percentage of radioactivity incorporated into phosphoglycerate revealed the steepest negative slope with time. Buchanan et al. nevertheless suggested that phosphoglycerate was formed as a result of the function of the reductive carboxylic acid cycle, perhaps mainly because they could not detect RuDP carboxylase in extracts. In our hands (F. R. Tabita and B. A. McFadden, unpublished data), RuDP carboxylase was readily detectable in the same strain now called *C. limicola* forma thiosulfatophilum strain 6230 (Tassajara) (kindly provided by N. Pfennig and grown in the absence of organic compounds), although the specific activity was low (7 nmol of CO_2 fixed per min per mg of protein at 30 C, pH 8.0, under optimal conditions) after transatlantic air delivery. In the face of the kinetic results at lower CO_2 concentrations and enzyme analyses in two different laboratories, it seems more likely that *Chlorobium* fixes CO_2 via the Calvin cycle, although this must be established more

rigorously. There is even some doubt that the reductive carboxylic acid cycle contributes to CO_2 fixation at the higher nonphysiological CO_2 concentrations used by Buchanan et al., because in studies with ^{14}C-succinate and 40 mM CO_2, malate and fumarate were the earliest labeled products and appeared much more rapidly than labeled glutamate plus glutamine (32). This is contrary to the expectation for function of the carboxylic acid cycle in a reductive direction.

Some enzymes of the reductive carboxylic acid cycle have been detected in *Chromatium* D (29) and *R. rubrum* (30), and its function has been postulated in these bacteria (57) on the basis that "they show amino acids as the main early products of $^{14}CO_2$ fixation". In support of the latter, the authors cited the work of Fuller et al. (62) and Hoare (87). In fact, experiments of Fuller et al. (62) established that *both* aspartate and 3-phosphoglycerate accounted for 70% of label fixed from $^{14}CO_2$ by *Chromatium* D in 30 s in the light and that key enzymes of the Calvin cycle were present at reasonably high levels —results in basic accord with those of Trüper with *Chromatium okenii* Perty (228).

Moreover, in more complete kinetic studies Hurlbert and Lascelles (94) convincingly established that autotrophically grown *Chromatium* D fixed CO_2 by the Calvin pathway. Of special significance, however, was the finding that growth on pyruvate favored subsequent CO_2 fixation into aspartate and suppressed the level of RuDP carboxylase. These results stress the influence of the mode of growth upon CO_2-fixing pathways. In this connection, the results of Hoare showing that glutamate and malate were major early products of CO_2 fixation were conducted in the light under H_2 with malate-grown *R. rubrum* (87). The function of the reductive carboxylic acid cycle in malate-grown *R. rubrum* does not even account for the labeling pattern of glutamate (Table 1) because it would have resulted in the rapid incorporation of ^{14}C from carboxyl-labeled acetate into C-3 and C-4 of glutamate, which was not observed. Quite recently Dhillon and Silver characterized nicotinamide adenine dinucleotide phosphate-specific isocitrate dehydrogenase from *R. rubrum* grown on malate in the light and found under a variety of conditions that the enzyme functioned only in the oxidative direction, which is the opposite direction from that required in the reductive carboxylic acid cycle (48, 57).

The function of pyruvate or phosphoenolpyruvate (PEP) carboxylase is required for acetate assimilation via the reductive carboxylic acid cycle (57). Wild-type *Rhodopseudomonas spheroides* lacks PEP synthase and carboxylase (165). Recently Payne and Morris (164, 165) characterized a mutant of this organism which was pyruvate carboxylase-less and would therefore not grow photosynthetically on glucose or pyruvate without addition of C_4-dicarboxylic acids. The mutant, however, grew photosynthetically on acetate and CO_2. This suggested that it could synthesize C_4-dicarboxylic acids by a route not involving carboxylation of pyruvate or PEP and thus could not be the reductive carboxylic acid cycle.

More recently, Beuscher and Gottschalk have failed to detect citrate lyase (or adenosine triphosphate [ATP]:citrate lyase), a key catalyst in the reductive carboxylic acid cycle, in extracts of *C. thiosulfatophilum* and *R. rubrum* examined under various conditions (22). Because the Göttingen group could readily detect the enzyme in extracts of *Rhodopseudomonas gelatinosa*, it seems unlikely that the former two organisms have a complete reductive carboxylic acid cycle.

To recapitulate, some photosynthetic bacteria have part of the enzymes of the reductive carboxylic acid cycle. However, there is no evidence for function of this cycle as a major CO_2 fixing path during strictly autotrophic growth, nor is there compelling evidence for function of the complete cycle during heterotrophic growth. The role and status of the reductive carboxylic acid cycle has also been recently reviewed elsewhere (176).

The Calvin cycle. Kinetic studies with *Rps. capsulata*, a photoorganotroph, established that CO_2 is fixed through function of the Calvin cycle (210). Therefore, it was of interest that Yoch and Lindstrom reported that *Rhodopseudomonas palustris* grown on CO_2 or formate in the light (4,000 ft-c) photoassimilated each substrate preferentially into amino acids in the shortest fixation time of 15 s. The composition of the medium in which cells had been suspended was not specified. Although supporting data were not provided, it was stated that the percentage of total radioactivity fixed into glutamate as a function of time showed a negative slope and that less than 6% of the radioactivity could be found in any of the phosphate esters. It was also stated, although exact growth conditions were not specified, that the Calvin cycle functioned predominantly during fixation of $^{14}CO_2$ in the light by malate-grown cells (247), a result in accord with that obtained for *Rps. isachenkoi* grown on malate in the light which, however, also fixed $^{14}CO_2$ rapidly into glutamate (50). The results of Yoch and Lindstrom

with autotrophically grown *Rps. palustris* are difficult to reconcile with those of Stokes and Hoare, who provided both kinetic data for $H^{14}CO_3^-$ assimilation (via $^{14}CO_2$) and enzymatic data that were compatible with the dominant function of the Calvin path in the same species suspended in phosphate buffer (209). Unfortunately, Hoare and co-workers did not specify the light intensity used during growth or fixation experiments (175, 209), so a direct comparison of their results with those of Yoch and Lindstrom (247) is somewhat difficult. Inferences that RuDP carboxylase functions in CO_2 fixation by photoheterotrophically grown rhodopseudomonads (50, 247) are compatible with the data of Lascelles, who observed increased levels of the enzyme in both *Rps. palustris* and *Rps. spheroides* after growth in the light in the presence of glutamate and malate (128) in comparison with those after growth with high aeration in the dark.

Rhodospirillum rubrum, a biotin-requiring organism that may be cultured in the light on CO_2 and H_2 photoorganotrophically or heterotrophically, affords an excellent organism to study the effect of growth conditions upon CO_2-fixing paths and has been the subject of several investigations. Working with cells grown on malate at unspecified light intensities, Glover et al. first established that 3-phosphoglycerate was the earliest major product of CO_2 fixation in the presence of H_2 with either high or low light intensities (69). Anderson and Fuller later provided extensive information on fixation of CO_2 by whole cells grown at a light intensity of 1,000 ft-c and suspended in growth media. The data suggested that: (i) the Calvin path dominated in cells grown on CO_2 and H_2 (6) and was important in cells grown on acetate in the light (7), and (ii) glycolate was the first labeled product of CO_2 utilization by cells grown on malate-CO_2 in the light (7). Although the mechanism of glycolate-labeling by CO_2 was not elucidated, enzymatic data were in basic accord with the observations on CO_2 fixation (8). For example, the specific activities of RuDP carboxylase and alkaline fructose-1,6-diphosphatase were drastically reduced by growth on malate compared with growth on CO_2 in the light, whereas values were intermediate after growth on acetate in the light.

Recently, Porter and Merrett have examined the influence of light intensity upon CO_2-fixing paths in acetate-grown *R. rubrum* (174). With cells grown at high light intensity (835 ft-c) the specific activity of RuDP carboxylase was 2.5-fold higher than after growth at low light intensity (140 ft-c). Indeed, kinetic experiments with cells assimilating acetate at high light intensity showed that high-light-grown cells incorporated $^{14}CO_2$ rapidly into phosphate esters in comparison with cells grown and fixing $^{14}CO_2$ at low light intensities. The percentage of total radioactivity present in phosphate esters plotted against time gave a negative slope for high-light conditions versus a positive slope for low-light conditions.

Quite recently, Slater and Morris have compared photoassimilation of CO_2 by batch and continuous-flow cultures of *R. rubrum* grown at a light intensity of 700 to 900 ft-c. In general, cells growing on malate fixed smaller but variable amounts of CO_2, depending on the stage and method of growth. The changing rates of photoassimilation of carbon dioxide paralleled changing specific activities of RuDP carboxylase (202).

Added bicarbonate is required for growth of non-O_2-evolving photosynthetic bacteria on fatty acids (234). It seems likely that some of these organisms use the fatty acids in conjunction with high light intensities to provide reducing power and energy for assimilation of CO_2 via the Calvin cycle. Of possible interest in this connection was the failure to find isocitrate lyase, a key enzyme of the anaplerotic glyoxylate cycle (117), in *R. rubrum* and *Rps. spheroides* grown on acetate either in the light or aerobically in the dark, although the enzyme was present in *Rps. capsulata* and *Rps. palustris* after growth under these conditions (119).

Because the blue-green algae are prokaryotic they will be examined in the context of photolithotrophic and photoorganotrophic bacteria, although they differ from other forms traditionally called photosynthetic bacteria in that they evolve oxygen during photosynthesis. In this latter, complex property of major physiological significance, the blue-green algae are similar to lower and higher plants. Early indications that the Calvin cycle functioned in CO_2 assimilation were obtained with $^{14}CO_2$ kinetic studies (155) and the finding that the labeling pattern (155) of polysaccharide-derived glucose was similar after fixation of $^{14}CO_2$ to that found for the green alga, *Chlorella pyrenoidosa* (115). Assimilation of CO_2 received renewed attention recently in studies of four strains of unicellular blue-green algae. Detailed studies of $^{14}CO_2$ fixation in the light and during a subsequent dark period after steady-state photosynthesis had been established were in complete accord with operation of the Calvin cycle (166).

In summary, the findings discussed establish that growth medium, light intensity, stage of

growth, conditions of CO_2 fixation, and, perhaps, the medium in which cells are suspended all influence CO_2-fixing paths in photoorganotrophic bacteria. Nevertheless, the evidence suggests that the Calvin cycle is the major assimilatory pathway during growth of all photosynthetic prokaryotic organisms on CO_2 as the main or sole carbon source.

Pathways in Chemolithotrophic Bacteria

Evidence suggesting the function of the Calvin cycle during fixation of $^{14}CO_2$ either by photoreduction in the presence of H_2 or by coupling with the hydrogen-oxygen reaction in the dark was first obtained with the alga *Scenedesmus* (15). Making allowances for a brief dark period after fixation but before extraction of cells by alcohol, negative slopes were obtained for the time course of incorporation of label into malate and organic phosphates consisting mainly of phosphoglycerate. Similar results were obtained after photosynthesis by this alga in the absence of H_2. Although the data did not rigorously rule out ^{14}C-malate as the precursor of ^{14}C-phosphoglycerate via turnover through fumarate to label C-1 and C-4 of malate, results indicated that two major paths of CO_2 fixation were occurring under all conditions of incorporation. One involved incorporation into C_4-dicarboxylic acids and the other involved the Calvin cycle. Thus in this alga the paths of CO_2 utilization were essentially identical in spite of coupling to different reductants and energy-trapping mechanisms. This was subsequently confirmed in more definitive experiments (67). Because assimilation of CO_2 driven by photoreduction with H_2 or the reaction of hydrogen with oxygen in the dark was also known to occur in bacteria, the need for research on autotrophic bacteria in that era was evident.

Among the chemolithotrophs, function of the Calvin cycle was first established for *Thiobacillus denitrificans* (147). Intact cells fixed $^{14}CO_2$ most rapidly into C-1 of phosphoglycerate, and other intermediates of the cycle were labeled as expected. In the absence of thiosulfate, cells took up 1% as much $^{14}CO_2$ (13). Subsequently, it was shown that the cycle dominated in CO_2 fixation by autotrophically grown *Hydrogenomonas facilis* not only in the presence of hydrogen and oxygen but also in the presence of nitrogen and oxygen, although fixation was reduced (137). (Davis et al. recently proposed a reclassification of polarly flagellated members of the hydrogen bacteria into the genus *Pseudomonas* (46). It was suggested that the peritrichously flagellated member, *H. eutropha*, be placed in *Alcaligenes*. In the final analysis, the validity of the proposal will depend upon genomic homologies. The obvious deoxyribonucleic acid (DNA) hybridization studies must still be completed, including one of DNA from *H. eutropha* with DNA from a type strain of *Alcaligenes* (180). To maintain historical clarity, the generic name *Hydrogenomonas* has been retained in the present review.)

Analogous studies at that time and since have revealed that the Calvin cycle is the major path of autotrophic CO_2 fixation in various species of *Hydrogenomas* (20, 86), *Thiobacillus thiooxidans* (221), formate-grown *Pseudomonas oxalaticus* OX 1 (177), *Micrococcus denitrificans* (118), *Nitrobacter winogradskyi* (113) and *agilis* (4), and probably in *Ferrobacillus ferrooxidans*, now called *Thiobacillus ferroxidans* (49).

In all of these genera, RuDP carboxylase and, in some cases, other enzymes of the cycle have been demonstrated in cell-free preparations after autotrophic growth (4, 49, 118, 142, 143, 154, 178, 196, 220, 227). Ribulose diphosphate carboxylase has also been demonstrated in extracts of *Nitrosomonas europaea* (181) and the marine chemolithotroph *Nitrocystis oceanus* (35). That it is a major protein in chemolithotrophic bacteria was indicated by the fact that it comprises up to 15% of the soluble protein in autotrophically grown *Hydrogenomonas eutropha* (B. A. McFadden and F. R. Tabita, in press).

In *H. facilis*, *H. eutropha*, *T. novellus*, and *T. ferrooxidans*, all facultative autotrophs, it is significant that heterotrophic growth under certain conditions drastically reduced the levels of RuDP carboxylase (5, 143, 154, 223), although the specific activity of phosphoribulokinase was much less variable in *H. facilis* (143).

Taken together, these observations made with diverse chemolithotrophic bacteria establish that the Calvin path dominates in assimilation of CO_2 by these microbes.

Regulation of the Calvin Cycle in Bacteria

Control of enzyme activity: by AMP. In 1965 Johnson and Peck discovered the inhibition by adenosine-5'-phosphate (AMP) of ribose-5-phosphate-, ATP-dependent CO_2 fixation in extracts of *T. thioparus* (99). They proposed that this phenomenon afforded a mechanism by which ATP could be conserved in times of "energy deprivation." Inhibition of autotrophic CO_2 fixation by AMP was subsequently observed with cell-free preparations of

H. facilis (142) and *T. novellus* (5), and evidence suggested that one locus of the inhibition was the step catalyzed by phosphoribulokinase (63, 133, 143). Although adenosine diphosphate (ADP) apparently also inhibited this step (98, 143), use of adenylate kinase-free enzyme will be required to establish this rigorously. Inhibition by AMP of ribose-5-phosphate-, ATP-dependent CO_2 incorporation was also observed in extracts of *Chromatium* D, spinach (98), and *Rps. spheroides* (187), and probably resulted from the inhibition of phosphoribulokinase. In contrast, partially purified phosphoribulokinase from *T. neapolitanus* was only slightly inhibited by 3.5 mM AMP (136). In the face of these varied reports, it is possible that regulation by energy charge (12) at this key step in the biosynthetic Calvin cycle is of importance in the "fine control" of this cycle in most but not all autotrophic bacteria. Another step that is inhibited by adenylate is catalyzed by 3-phosphoglycerate kinase of *H. facilis* (141).

By NADH. A major difference in carbon metabolism between lithotrophic bacteria and O_2-evolving photosynthetic species (including blue-green algae) is the use by the former of reduced nicotinamide adenine dinucleotide (NADH) as the reductant of CO_2 instead of reduced nicotinamide adenine dinucleotide phosphate, as was stressed by McFadden and Tu (142, 143).

These workers observed ATP- NADH-dependent CO_2 fixation catalyzed by the high-speed supernatant fraction from extracts of *H. facilis*. This Mg^{2+}-dependent incorporation of CO_2 was inhibited by AMP. These and other properties and the fact that noncyclic incorporation would have required a total endogenous concentration of acceptor(s) of 10 to 15 mol per liter (143) led to the conclusion that fixation had occurred via the Calvin cycle. Indeed, the specific activities for this fixation correlated well with that of RuDP carboxylase but not phosphoribulokinase. This interpretation was later challenged by MacElroy et al. on the basis of the important finding that NADH was a positive allosteric effector of phosphoribulokinase from *H. facilis* (135). Considering both lines of evidence, it is obvious that NADH may function in CO_2-fixing extracts both stoichiometrically as a reductant of CO_2 and as a positive effector of phosphoribulokinase. The latter phenomenon may certainly contribute to acceleration of carbon flow through the Calvin cycle, thereby tending to lower the concentration of reductant.

A recent report has suggested that NADH is also a positive effector of phosphoribulokinase from *Rps. spheroides* (187), whereas NADH at 3.5 mM has no effect upon the enzyme from *T. neapolitanus* when assayed under optimal conditions (136). It will be important to ascertain the natural distribution of this stimulation by NADH among lithotrophic bacteria.

By other metabolites. Because the Calvin cycle provides intermediates for all biosynthesis in lithotrophic bacteria, the possibility of elements of positive and negative control exerted through metabolites other than NADH and ATP has been evident for some time. The first convincing example of this was a recent report of the highly specific inhibition by PEP of phosphoribulokinase from *H. facilis* (16). Binding and resultant inhibition by PEP was strongly cooperative at saturating substrate concentrations and the apparent K_i was 0.55 mM. Although the phosphoribulokinase from *T. neapolitanus* was less sensitive to PEP, synergistic inhibition (83%) was observed in the presence of 2.3 mM PEP plus 3.5 mM AMP (136). Thus PEP, which is a metabolite of central importance and is almost directly derived from the Calvin cycle, may contribute to regulation of the cycle in lithotrophs.

More recently, striking inhibition by 6-phosphogluconate of RuDP carboxylases (K_i's of about 0.1 mM) from *Chromatium* D, *Ectothiorhodospira halophila*, an extremely halophilic photolithotrophic bacterium, *H. eutropha*, *Anacytis nidulans*, and spinach has been reported (222). Similar results for the spinach enzyme were published almost simultaneously (39). In this connection, in *Chlorella pvrenoidosa*, Bassham and Kirk used both ^{32}P-labeled phosphate and ^{14}C-labeled CO_2 to show that the light-dark transition was accompanied by the appearance of labeled 6-phosphogluconate in the dark (19). This was taken to reflect the function of the oxidative pentose phosphate cycle and indeed all enzymes of the oxidative phosphate cycle were known to be present in isolated chloroplasts (80). It was also demonstrated that pentose monophosphates could be converted to RuDP in the dark, yet isolated chloroplasts could not catalyze carboxylation of RuDP in the dark (19). On the basis of these results, it seems likely that 6-phosphogluconate is the regulator that slows carboxylation in the dark in green plants.

Phosphogluconate is also an important intermediate in the heterotrophic metabolism of the hydrogen bacteria. This intermediate is catabolized through the Entner-Doudoroff path in these bacteria (28, 71, 122). Thus the levels of "free" phosphogluconate in the cell may reflect the function of this path and inhibit the Calvin

cycle at RuDP carboxylase, an enzyme present at high levels in addition to phosphoribulokinase in sugar-grown cells (143). It should be noted that *Anacystis nidulans* also has the enzymes of the pentose phosphate path (185); presumably, 6-phosphogluconate, which is known to accumulate in the dark (166), regulates RuDP carboxylase (10) in this organism as well.

Other potential loci of regulation in the Calvin cycle are the steps involving the hydrolytic removal of phosphate from the 1,7- and 1,6-diphosphates of sedoheptulose and fructose. It is thought that each of these enzymes functions uniquely in the Calvin cycle in plants and indeed the activities were separated some time ago (179). In bacteria the situation may be different, however. Quite recently, fructose-1,6-diphosphatase was purified to homogeneity from *Rps. palustris* that had been grown in the light on a malate-amino acid mixture. The V_{max} for sedoheptulose-1,7-diphosphate was 22% of that for fructose-1,6-diphosphate, and the K_m was 3.6-fold greater. Unfortunately, the activity towards these two substrates was not compared throughout purification (204). Nevertheless, the results suggest that there may be only one diphosphatase in *Rps. palustris*. It would be interesting to compare the two species of this enzyme (mol wt 65,000 and 130,000), which are reversibly interconverted as a function of pH with regard to catalytic specificity (206). The smaller, more active form is favored under more alkaline conditions. The basis for the curious inhibition of this enzyme by nucleoside triphosphates (205) merits further examination as it is contrary to the expectation for a step in a biosynthetic sequence.

Control of enzyme synthesis. Presumably, three enzymes function uniquely in the Calvin cycle during growth of lithotrophic bacteria: RuDP carboxylase, phosphoribulokinase, and alkaline fructose-1,6-diphosphatase, which may function also as sedoheptulose-1,7-diphosphatase. Little detailed work has been done on synthesis of the diphosphatases, although, as mentioned previously, growth of *R. rubrum* on malate-CO_2 in the light drastically reduced the level of alkaline fructose-1,6-diphosphatase compared with the level after growth on H_2-CO_2 (7). Work on sedoheptulose-1,7-diphosphatase has undoubtedly been hampered by the difficulty in preparing the pure substrate (179). Nevertheless, it will be important to assess the diphosphatase activities in facultative lithotrophic bacteria to determine whether they are due to a single enzyme and whether their synthesis is repressed by heterotrophic growth as is the case for RuDP carboxylase under most conditions.

Of interest is the possibility that structural genes specifying the structure of these proteins comprise an operon (95) in lithotrophic bacteria. Coordinated control of the synthesis of these catalysts would be of obvious advantage. In this connection, ratios of specific activities of RuDP carboxylase and phosphoribulokinase in *H. facilis* are quite variable after various modes of growth as evident from Table 2, which was taken from the data of McFadden and Tu (143). The data suggest, but do not prove, that the synthesis of these enzymes is not coordinately regulated in *H. facilis*. The relevant genetic work should be done on this and other organisms. One approach would be to isolate isocitrate lyase-negative mutants of *H. facilis* which, unlike their wild-type parents (139), would not grow on acetate. The autotrophic growth of these mutants would presumably be strongly inhibited by inclusion of acetate in the growth medium, as would also be the case for the wild-type parents. From these mutants, it may be possible to derive mutants that reveal acetate-resistant autotrophic growth and are therefore constitutive for production of one or both enzymes in question.

The data in the right-hand column of Table 2 are also of interest. The evidence that the reductive, ATP-dependent fixation of CO_2 by extracts occurred via the Calvin cycle has been discussed in an earlier section. As evident, these data revealed a reasonably good correlation with the specific activity of RuDP carboxylase but not that of phosphoribulokinase. The supporting arguments that the levels of RuDP

TABLE 2. *Specific activities of RuDP carboxylase, phosphoribulokinase, and ATP-, NADH-dependent CO_2 fixation in H. facilis*

Growth substrate	Specific activity[a]		
	RuDP carboxylase	Phosphoribulokinase	ATP-NADH-dependent CO_2 fixation
CO_2	67	20	4.6
Fructose	51	22	4.8
Glucose	51	21	3.1
Ribose	25	12	2.4
Glutamate	2	13	0.9
Lactate	10	13	1.6
Succinate	2	13	0.2
Acetate	1	2	0.4

[a] Expressed as nanomoles of product per minute per milligram of protein.

carboxylase in these extracts limited the rate of CO_2 fixation via the Calvin cycle have been presented elsewhere (143). As would be expected, ribose-grown cells fixed a smaller fraction of $^{14}CO_2$ into phosphoglycerate when suspended in ribose medium (140).

Recently Joint et al. (102) obtained a complex of alkaline fructose-1,6-diphosphatase and phosphoribulokinase from autotrophically grown *R. rubrum*. By the criteria of isoelectric focusing and polyacrylamide gel electrophoresis, the complex was pure. Loss of both activities with storage seemed to correlate with the appearance of two gel electrophoretic components. Although these two enzymes do not catalyze sequential reactions in the Calvin cycle, isolation of the complex is almost certainly not fortuitous. It raises the question of the possible combination of this complex with other complexes, as yet unidentified, to construct an organized array of enzymes which function in the Calvin cycle. Moreover, it elevates interest in the regulation of alkaline fructose-1,6-diphosphatase and phosphoribulokinase.

Control by other mechanisms. The regulation of RuDP carboxylase has proven to be complex in *H. facilis* (123). When this organism was cultured on fructose with high aeration, synthesis of the enzyme was totally repressed. In contrast, growth with lower aeration in iron-supplemented medium resulted in RuDP carboxylase-rich cells until the late-log phase. Upon depletion of fructose, carboxylase activity decayed rapidly and the decay could be triggered in cells harvested in the mid-log phase and then deprived of fructose. The latter decay had a half-time of about 20 min at 30 C. A similar decay was observed under certain conditions in extracts of fructose-grown cells but not in extracts of autotrophically grown cells. Although several lines of indirect evidence suggested that proteolysis was responsible, the apparent requirement of ATP for inactivation of RuDP carboxylase was reminiscent of the enzyme-catalyzed, ATP-requiring adenylation of glutamine synthetase (for a review see 207).

Crystallization of RuDP carboxylase of lower specific activity and lower absorbancy ratio at 280 and 260 nm from *H. facilis* (124) raises the intriguing possibility that this enzyme is regulated by AMP- (or ADP-)amplified adenylation, at least during heterotrophic growth. A possibly analogous decay of activity of this enzyme was noted by Hurlbert and Lascelles when autotrophically grown cells of *Chromatium* D were transferred to pyruvate medium (94).

A two- to threefold increase in total activity of RuDP carboxylase was observed during purification of the enzyme in extracts of *T. denitrificans* (138) and *H. facilis* (124). The data, which were obtained with dialyzed fractions, suggest the occurrence of natural macromolecular inhibitors in some autotrophic bacteria. Whatever the correct interpretations of these phenomena prove to be, inactivation of RuDP carboxylase may contribute significantly to the regulation of autotrophic CO_2 assimilation. In this regard, it will be important to compare the effects of partial inactivation upon maximal velocity and the K_m.

Comparisons with green plants. The regulation of CO_2 assimilation by green plants has been recently reviewed by Bassham (18). Experimental approaches discussed included the alteration of metabolite pool sizes after achieving steady-state photosynthesis. Perturbation has been achieved by dark periods or the addition of compounds such as octanoate and vitamin K_5. With the latter, for example, it was possible to mimic the dark-induced accumulation of 6-phosphogluconate described previously. In addition, use of synchronous algal cultures was described. By use of cultures at different stages of the division cycle and detailed studies of metabolite variations during the approach to steady-state photosynthesis in each, specific sites of regulation of carbon metabolism in the light were tentatively identified. These were (i) steps catalyzed by fructose-1,6-diphosphatase and RuDP carboxylase, (ii) the synthesis of sucrose, probably at the stage of sucrose phosphate synthesis, and (iii) the conversion of PEP to pyruvate. The power of these experimental approaches with intact cells or chloroplasts is self-evident, but as yet little has been done with the lithotrophic bacteria along these lines.

A recent observation may be relevant. Buchanan and colleagues found that highly purified, spinach alkaline fructose-1,6-diphosphatase was strongly stimulated by a partially purified protein and reduced ferredoxin (31). It was suggested that the activity of fructose-1,6-diphosphatase may be increased indirectly by the level of reduced ferredoxin, thereby providing a link between light and dark reactions. It will be important to probe for an analogous stimulation in the photolithotropic bacteria.

Of particular regulatory significance may be the effect of 10 mM Mg^{2+} in lowering the pH optima of the plant but not bacterial RuDP carboxylases from the alkaline side into the range of 6.5 to 7.0 (217, 218), if the observed shift proves to be independent of the buffering species. Sugiyama et al. speculated (218) that activation of RuDP carboxylase by Mg^{2+} may

be linked to the light-induced pH decrease within chloroplasts accompanied by a change in chloroplast structure (96, 162).

EVOLUTION OF AUTOTROPHIC CO_2 FIXATION

Chemical Evolution and the First Organism, a Heterotroph

The concept that organic compounds which serve as the basis of life were formed when the earth had a reducing atmosphere of ammonia, hydrogen, methane, and water vapor was first suggested in a booklet by Oparin in 1924, although this postulate did not become widely known until 1938 (159). Since then, dating from the experiments of Miller in 1953 which established that amino acids could be produced by passing an electrical discharge through this atmosphere, numerous chemical syntheses have been described under conditions simulating those of primitive earth. These have been reviewed recently (34, 129, 172) and will not be reiterated in detail here. Suffice it to say that amino acids, purines, pyrimidines, sugars, fatty acids, porphyrins, proteins, and obligonucleotides have been produced under such conditions. Thus it is likely that chemical evolution produced a complex array of compounds between the formation of the earth about 4.7×10^9 years ago and the appearance of the first organisms approximately 1.6×10^9 years later (34, 129). This interval is based in part upon recent descriptions of unicellular algal and bacterial fossils from the Pre-Cambrian period (17, 56, 197) and may, of course, prove to be shorter with future paleo-ontographical evidence.

Oparin in 1924 (159) and, independently, Haldane in 1929 (73) were the first to postulate the emergence of life in the presence of diverse organic compounds that had accumulated on the prebiotic earth, especially in the oceans. Thus it was recognized that the first living entity probably had little or no biosynthetic capacity.

Retroevolution

A plausible mechanism for the evolution of catalysts in biosynthetic sequences remained an enigma until 1945. Horowitz recognized the improbability of the development of sequences enabling the eventual conversion of early intermediates to products which were required for growth by emphasizing that each acquired step would have been selectively worthless and therefore unlikely to persist (92). As an alternative, with the premise that prebiotic environments contained all compounds to be utilized eventually in biosynthesis in descendant organisms, Horowitz postulated that the development of biosynthetic paths occurred by a process often referred to as retroevolution. To illustrate, we may consider the evolutionary development of the biosynthesis of an amino acid by a pathway now known to be:

$$A \xrightarrow{a} B \xrightarrow{b} C \xrightarrow{c} D \xrightarrow{d} E \xrightarrow{e} \text{amino acid}$$

It was postulated that depletion of the essential nutrient (in this case an amino acid) by a primitive organism accompanied by the gain of an enzyme which catalyzed the conversion of E to the amino acid would have conferred an enormous selective advantage upon that organism. By carrying this hypothesis to its logical conclusion, the evolution of entire biosynthetic sequences was envisaged.

The hypothesis of Horowitz was later enlarged by Lewis, who suggested that duplication of the ancestral gene specifying the terminal catalyst (e in the model pathway) would have resulted in a redundant gene which might then have been modified to specify a protein (d in the model pathway) that catalyzed the formation of the penultimate compound in the pathway (130). In its logical conclusion, retroevolution of all structural genes in a pathway would then have occurred from a common ancestral origin (that specifying e) by tandem duplication and mutation of each resultant gene. This is easier to visualize when it is remembered that a given postulated precursor gene already specified the three-dimensional binding site for a substrate that was to become a product of the preceding reaction in the growing pathway. Thus mutation had only to result in the modification of an existing binding site and acquisition of new sites and catalytic capacity.

The views of Lewis were further refined by Horowitz, who pointed out that the existence of operons—adjacent, coordinately regulated units of functionally related genes—accounted for the hypothesized tandem gene duplication and modification (93). He also emphasized that evolution by this mechanism should have resulted in detectable homologies in sequentially functioning proteins specified by an operon. Hegeman and Rosenberg have recently reviewed the limited data that bear on this point (81) and which provide no evidence for the predicted homologies.

There is one line of direct evidence compatible with retrograde evolution. Lin and co-workers (131, 246) showed that xylitol transport was gained by "fitter" mutants of *A. aerogenes* growing at the expense of xylitol after the initial

acquisition of a dehydrogenase which catalyzed conversion of xylitol to xylulose.

In conclusion, the theory of retroevolution *as originally promulgated* by Horowitz in 1945 (92) is very attractive in accounting for the evolution of biosynthetic paths. For a given path, the initiating step was acquisition of the terminal catalyst. This catalyst, and subsequently appearing ones as well, might have arisen from mutation of genes specifying structures of enzymes of similar function in other pathways. Utilization of pre-existing structural genes, however, begs the question of the origin of the most primitive biosynthetic paths. Perhaps only in those paths will homologies between functionally related proteins be found.

From Heterotrophism to Autotrophism

Accepting the reasonable premise that the most primitive organism was a heterotroph with limited biosynthetic capacity, we may ask how autotrophs appeared. Presumably the predecessors were anaerobic and the gain of assimilation of CO_2 superimposed upon a heterotrophic metabolism was of selective value. This need not have meant that such organisms were capable of growth under strictly autotrophic conditions but only that they acquired enzymes enabling use of the Calvin cycle to complement heterotrophic assimilatory paths. Two nonphotosynthetic organisms that develop anaerobically and which obtain energy from the oxidation of inorganic matter are *Micrococcus denitrificans* and species of *Desulfovibrio*. Rittenberg has reviewed experiments which suggested that *D. desulfuricans* utilizes CO_2 and yeast extract simultaneously when oxidizing hydrogen with sulfate (188). It will be important to examine this species for the presence of RuDP carboxylase and phosphoribulokinase. It is of interest that the emergence of sulfate-reducing bacteria has been dated between 2 and 3.5 billion years ago on the basis of geochemical evidence (14). Contrary to these implications is the recent finding that one-half of the amino acid sequence of ferredoxin from *D. gigas* is similar to a portion of ferredoxin from higher plants, whereas the other half is similar to half of the molecule from several bacteria including *Chromatium* (225). In the latter organisms, the second half shows a high degree of homology to the first, suggesting that the ancestral structural gene for ferredoxin doubled during evolution. These data suggest, but do not prove, that *D. gigas* is more advanced than *Chromatium*.

With regard to *M. denitrificans*, strictly autotrophic growth on H_2, O_2, and CO_2 is accounted for by function of the Calvin cycle (118). The requirement of yeast extract when nitrate is substituted for oxygen merits further examination (188). If this organism can utilize both CO_2 and some compounds in the yeast extract during the oxidation of H_2 by nitrate, it may be relatively primitive among chemolithotrophs.

Quite recently, the isolation and characterization of *Methanobacterium thermoautotrophicus* has been described (248). Growth of this organism at 65 to 70 C on H_2, CO_2, and minerals required only the addition of vitamins. Cysteine stimulated the growth by an unknown mechanism. Ribose-5-phosphate-, ATP-dependent CO_2 fixation was detected in extracts, although the specific activity was not measured nor was its contribution to CO_2 assimilation established (J. G. Zeikus, personal communication). If this organism utilizes the Calvin cycle, it may be representative of very primitive autotrophism. In this connection, Sagan has estimated that stabilization of the earth's mantle occurred 4.5×10^9 years ago (194). Harada and Fox have suggested that when the earth was formed, the gravitational energy was converted to thermal energy and the temperature of the crust should have increased to about 1,000 C (74). Thus, as the surface cooled the initial temperature of the developing oceans may have been elevated. Volcanic action also should have supplied thermal energy. Enzymes and ribosomes of thermophilic bacteria are known to have unusual thermal stability. Of interest also are the observations that as the growth temperature increases the nutritional requirements generally increase (58). To this reviewer, the evolution of mesophiles from thermophilic ancestors is more sensible than the converse. It is easier to envision the gradual loss of thermal stability as the environment cooled because, in general, macromolecules would have functioned reasonably effectively without mutational alteration during the transition. Thus random mutations might have gradually been fixed resulting in the selection of macro-molecules that were "fitter" in functioning at lower temperatures. In the light of these considerations, *M. thermoautotrophicus* may have descended from a very primitive mode of autotrophism.

Other lithotrophic organisms, however, are also facultative and anaerobic and may therefore reflect primitive autotrophic life. These are the photolithotrophic bacteria, and some of their properties have been described in an earlier section. Of particular interest in this group are members of *Rhodospirillum* and *Rhodopseudomonas*. They are facultative and use the Calvin cycle for growth on CO_2, which usually requires the addition of organic growth

factors. However, these organisms are putatively more advanced than their anaerobic chemolithotrophic counterparts because of their capacity to trap radiant energy.

Finally, among the bacteria are organisms which grow in the presence of H_2 on (i) CO_2, (ii) methanol plus bicarbonate, or (iii) formate plus bicarbonate. The properties of these organisms, which produce CH_4 and also may grow on short-chain alcohols and fatty acids, have been reviewed (176). The pathway of net carbon assimilation by these bacteria, which are members of *Methanosarcina*, *Methanococcus*, and *Methanobacterium*, is unknown but of obvious interest.

Given that anaerobic autotrophs developed before aerobic forms, the sequence of appearance of photolithotrophs which evolve O_2 versus chemolithotrophs may next be considered. Most of the latter organisms are obligately aerobic and include one or more species of *Hydrogenomonas*, *Pseudomonas*, *Nitrobacter*, *Nitrosomonas*, *Nitrocystis*, and *Thiobacillus*. If O_2 in the ancient atmosphere was of biological origin, the aerobic chemolithotrophs must have descended from O_2-evolving species. Although the view that O_2 originated as a result of photosynthesis perhaps prevails (160), the alternative of its formation from photodissociation of water should not be overlooked (195).

In addition to CO_2, a number of one-carbon compounds varying in oxidation state from methane to formate can be assimilated aerobically by bacteria. The assimilatory paths have been recently reviewed (176). The three main routes described are (i) a serine path, (ii) a path involving *N*-methylated amino acids, and (iii) a ribose-phosphate cycle involving formaldehyde fixation. The reader should consult the excellent review by Quayle for details (176). Quayle has suggested a possible evolutionary development of methane utilization from ammonia-oxidizing lithotrophy (176). Although there is as yet no obvious relationship between these interesting paths of methane utilization and that used by predominant forms of autotrophic life, further work on the enzymology of the formaldehyde-fixing cycle, especially, is of obvious interest.

New Catalysts in CO_2-Fixing Autotrophs

RuDP carboxylase and phosphoribulokinase. The role of enzymes which function uniquely in the Calvin cycle has been discussed in a previous section. The two enzymes which have no counterparts with closely analogous function are RuDP carboxylase and phosphoribulokinase. Thus a consideration of the evolution of this cycle basically reduces to a consideration of these two catalysts. Glycolysis is widely distributed in anaerobic bacteria and glucose-6-phosphate plus 6-phosphogluconate dehydrogenases, pentose phosphate isomerases, transketolase, and transaldolase occur in some, if not most, anaerobes (245). These observations suggest that glycolysis and the oxidative hexose monophosphate path were established in reasonably primitive organisms and that catalysts functioning in each pathway were available intracellularly during acquisition of the carboxylase and kinase.

If in a specific, ancient ecological habitat the availability of hexoses became limiting, organisms that acquired the capacity to convert CO_2 to hexoses via the Calvin cycle should have had a marked competitive advantage. The obvious dilemma in this conjecture is that the acquisition of both RuDP carboxylase and phosphoribulokinase would have been required to close the cycle. If, however, one of these two catalytic activities already resided in another enzyme—albeit weakly—at the time of appearance of the second enzyme, the organism should still have been competitive. In this connection, the most obvious enzyme that might have shown some activity towards phosphoribulose is phosphofructokinase. Although the substrate of the latter enzyme is probably the furanose, whereas phosphoribulose is acyclic, it is perhaps significant that fructose-1,6-diphosphate is a good competitive inhibitor with respect to RuDP for RuDP carboxylase (see section **Inhibition** and reference 26). Therefore, an interesting precedent exists for apparent competition between these cyclic and acyclic sugar phosphates. It would thus be of interest to examine the specificity of bacterial phosphofructokinases and phosphoribulokinases with respect to phosphorylation of fructose and ribulose phosphates. It is easy to imagine the evolution of a fitter phosphoribulokinase from the genome specifying phosphofructokinase after the establishment of RuDP carboxylase. No similarly close enzymatic analog of RuDP carboxylase exists and it is tempting to consider that appearance of the carboxylase triggered the evolution of efficient autotrophic CO_2 fixation.

Properties of RuDP Carboxylase

As evident from the previous sections, discussions of the sequence of appearance of autotrophic forms of life in evolution are quite tentative. To attack the issue experimentally requires the examination of a property or properties shared by all autotrophic species. The most obvious single property is that of CO_2 fixation, the primary step of which is catalyzed

by RuDP carboxylase which is ubiquitous among autotrophs.

Much is known of the structure and function as well as regulatory properties of this enzyme, but almost nothing is known about these properties of phosphoribulokinase. RuDP carboxylase was the subject of a review by Kawashima and Wildman in 1970 (107), but considerably more information is now available. Accordingly the properties of RuDP carboxylase will be examined in the next section.

Kinetic response to substrates. Concern still persists as to whether RuDP carboxylase actually catalyzes primary CO_2 fixation because the K_m for CO_2, which is now known to be the substrate (45), is considerably higher (107) than equilibrium concentrations of CO_2 in the aqueous milieu of autotrophic species. For example, Hatch and Slack recently emphasized (78) that for spinach RuDP carboxylase the K_m for CO_2 of 0.45 mM (44) is too high to account for CO_2 fixation, because the "concentration of CO_2 in a solution in equilibrium with air and at temperatures between 20 and 30 C would be about 8 μm irrespective of pH." Invocation of the function of chloroplastic carbonic anhydrase to concentrate CO_2 (78) may be fruitless because the concentration of CO_2 plus HCO_3^- in the chloroplast cannot exceed the extra-chloroplastic concentration of these species unless there is a chloroplastic mechanism to pump in HCO_3^- (or related species). To this reviewer, the most plausible explanation for the discrepancy between the K_m and (CO_2) is that the K_m does not reflect the true K_m in situ. That this may well be the case was strongly indicated by the finding with intact spinach chloroplasts that the K_m for fixation of HCO_3^- (or CO_2) was about 20-fold smaller than that for isolated RuDP carboxylase and that the fixation occurred by the Calvin path (97). Moreover, the maximal rate of photosynthesis by these freshly prepared chloroplasts was 63% of that of fresh spinach leaves. These results suggest that the kinetic properties of RuDP carboxylase are altered by removal from its natural environment. This may be true for the bacterial enzymes as well because they also have high K_m values for CO_2 (107). These considerations imply that RuDP carboxylase functions in vivo at concentrations of CO_2 which are well below that required for saturation.

As mentioned in a previous section, RuDP carboxylase from *Hydrogenomonas* comprises up to 15% of the "soluble" protein. Although it has not been purified to homogeneity from CO_2-grown *R. rubrum* or *T. denitrificans*, it can be estimated that the carboxylase is about 6 and 2%, respectively, of the "soluble" protein from these two sources (9, 138). The enzyme is undoubtedly a major protein in chloroplasts of higher plants (107). For example, it is 16% of the "soluble" protein in spinach leaves (163). The presence of such large amounts of a catalytic protein in leaf tissue has led to speculation about additional functions of RuDP carboxylase (107). It seems likely to this reviewer, however, that the large amount present in this tissue and other sources as well more probably reflects its inefficiency as a catalyst. The turnover numbers, which are proportional to specific maximal velocities, of RuDP carboxylase in terms of moles of CO_2 fixed per second per mole of catalytic site at 30 C are 1 and 2 for the *Hydrogenomonas* and spinach enzymes, respectively. This estimate assumes 12 and 8 catalytic sites per molecule of enzyme (mol wt 500,000), respectively (107, 124). These turnover numbers are 5- to 10-fold less than the smallest values in the range of 10 to 10^7 tabulated in a recent book (21). The enigma is why such a comparatively inefficient catalyst became established during evolution, although it must be emphasized that the enzyme-catalyzed reaction is probably at least 10^9 faster than the uncatalyzed nonenzymatic reaction (169).

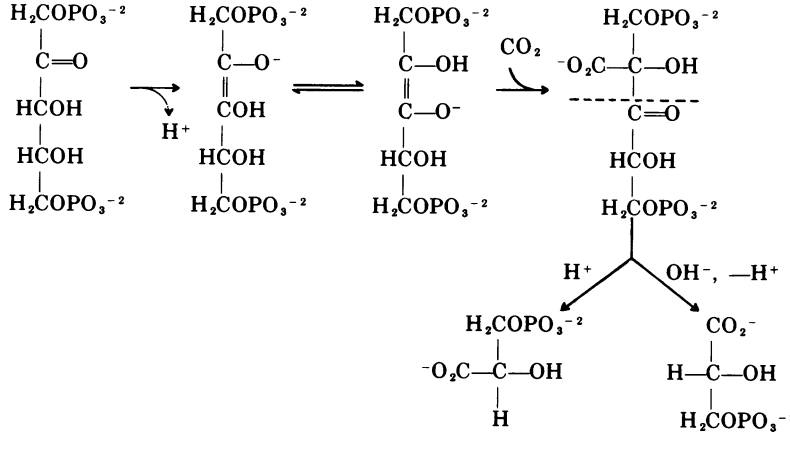
It has become apparent from the contributions of Akazawa and his colleagues that the binding of substrates by RuDP carboxylase may be weakly homotropic or heterotropic, depending upon the source of the enzyme and the concentration of Mg^{2+}. In general, studies of the enzyme from *Chlorella* (217) and spinach (218) revealed positive homotropism for HCO_3^- or CO_2 (152) binding. Added Mg^{2+} lowered the apparent K_m or $(S)_{0.5}$, i.e., the substrate concentration at which the velocity was half-maximal (121). In contrast, studies of the enzymes from *Rps. spheroides* and *R. rubrum* (2) also revealed homotropic interaction with HCO_3^- but no effect upon $(HCO_3^-)_{0.5}$ by added Mg^{2+}. Moreover, homotrophic interaction with RuDP that vanished with added Mg^{2+} was revealed. The $(S)_{0.5}$ for RuDP in the presence of added Mg^{2+} was about 0.2 mM, which is within the normal range observed for enzyme from diverse sources (107). Whereas cataloging of these ligand-enzyme interactions will certainly be of interest in comparing RuDP carboxylases, it should be emphasized that the interaction coefficients (n values in the Hill equation, see reference 11) did not in any case exceed 2 (2, 217, 218), whereas with the plant enzyme the limiting value would be 8 for infinite cooperativity if it is assumed that each large subunit has one catalytic site (see "**Quaternary structure etc.**" and reference 107). Thus the cooperativities observed were small and may be of

minor significance in the "fine control" of RuDP carboxylase. However, the effect of 10 mM Mg^{2+} in lowering the pH optima of the plant but not bacterial enzymes from the alkaline side into the range of 6.5 to 7.0 (217, 218) may be of greater physiological significance as mentioned previously.

Metal ion requirement. Kawashima and Wildman have discussed the substitution of Mg^{2+} by Ni^{2+} or Co^{2+} for enzyme from some sources. In general, the efficacy depends upon age and purity of the enzyme. Generally, RuDP carboxylase activity is highly dependent upon added Mg^{2+} (107). Of interest, then, was the report of Anderson and Fuller that the highly purified enzyme from *R. rubrum* showed no dependence upon added Mg^{2+} (9), although 0.1 mM Mg^{2+} was apparently present during assay. In those studies highly purified enzyme was treated briefly with 1 mM ethylenediaminetetraacetic acid (EDTA) and then filtered on gel which had been treated with EDTA for 3 days and washed with "metal-free" tris(hydroxymethyl)aminomethane buffer. The same buffer was used for elution but unfortunately contained no EDTA. These studies do not rule out a dependence upon Mg^{2+} but do suggest that the enzyme from *R. rubrum* either has an unusually high affinity for Mg^{2+} or that dissociation of Mg^{2+} is kinetically very slow. The K_m for Mg^{2+} is 1.1 and 1.4 mM for the higher plant and hydrogenomonad enzymes, respectively (107, 124). In contrast to the results of Anderson and Fuller are those of Akazawa et al., who observed 50% activation of RuDP carboxylase (of unknown purity) from *R. rubrum* by added saturating Mg^{2+} (5 mM). As yet, there is no explanation for this discrepancy, although the *R. rubrum* enzyme is far less responsive to added Mg^{2+} than is the spinach enzyme (2, 218).

Specificity and mechanism of catalysis. Until quite recently, available evidence suggested that RuDP carboxylase was highly specific for the substrates CO_2 and RuDP. However, in 1971 Ogren and Bowes established that O_2 was a competitive inhibitor with respect to CO_2 for the soybean enzyme (156), leading to the recognition that the enzyme actually functioned at higher partial pressure of O_2 in the cleavage of RuDP. The products were phosphoglycolate (27), derived from the top two carbons of RuDP, and phosphoglycerate derived from C-3, C-4 and C-5 (N. E. Tolbert, in Algal Physiology and Biochemistry, in press). Accordingly, the name RuDP oxygenase has been suggested for this activity (N. E. Tolbert, in press). Although phosphoglycolate production was demonstrable after incubation of soybean or corn leaf extracts with RuDP under air or O_2 (27), little is known of the natural distribution of phosphoglycolate generation by RuDP carboxylases. This is of significance because of conjecture that phosphoglycolate production during photorespiration of plants is due to catalysis by the oxygenase component of RuDP carboxylase (156; N. E. Tolbert, in press). Although the precise role and degree of this alternative in catalysis for RuDP carboxylase in vivo remains to be established, it may account, in part at least, for the unusually large amounts found in plants. Furthermore, a hitherto unrecognized function of RuDP carboxylase may explain why the enzyme is present at high levels under certain conditions of heterotrophic growth of bacteria (123, 143, 223). It will be of obvious importance to probe for oxygenase activity among bacterial RuDP carboxylases.

With regard to the mechanism of action, the following pathway is consistent with the experimental data:



Kawashima and Wildman have recapitulated (107) the proof by Rose and colleagues (59, 151) that the CO_2 addition occurs at C_2 and that tritium is lost to the medium by C_3-tritiated RuDP during catalysis by RuDP carboxylase. On the basis of the finding that RuDP may also undergo oxygenolysis to phosphoglycolate and phosphoglycerate, oxygen and water presumably add to the double bond of the 2,3-enediol intermediate such that ^{18}O from molecular oxygen appears in phosphoglycolate, whereas that from water appears in phosphoglycerate (N. E. Tolbert, in press).

The reaction mechanism may be ordered on the basis that preincubation of the enzyme with Mg^{2+} followed by bicarbonate with subsequent initiation of the reaction by RuDP yields a much higher rate of CO_2 fixation than do other combinations and sequences of substrate addition (124, 138, 171). Thus it has been inferred that the preferred order of addition to the enzyme from *Tetragonia*, *Hydrogenomonas*, and *Thiobacillus* is Mg^{2+}, CO_2, and RuDP. However, these experiments should be repeated in the presence of exogenous carbonic anhydrase since they may only reflect the time dependence of equilibration of the substrate, CO_2, with provided bicarbonate (44). In this connection it is of some interest that carbonic anhydrase has been recently detected in one of the thiobacilli (190). For elucidation of the kinetic mechanism, a detailed examination of product inhibition patterns may be fruitful (40).

Inhibition. The anions, orthophosphate and sulfate, are competitive inhibitors with respect to RuDP for RuDP carboxylase from spinach and *H. facilis* although the K_i's are 4 to 10 mM (124, 163). In contrast, sulfate has no effect upon the enzyme from *H. eutropha* and orthophosphate is stimulatory with enzyme from that source (124) and slightly inhibitory with enzyme from *R. rubrum* (9). The effects of a number of plausible metabolic inhibitors of RuDP carboxylase have been examined. Citrate appears to be a competitive inhibitor with respect to RuDP (K_i = 0.047 mM) for enzyme for *R. rubrum* (9), although a physiological role for this inhibition remains to be established. 3-Phosphoglycerate is a competitive inhibitor with respect to HCO_3^- and noncompetitive with respect to RuDP for enzyme from *H. facilis* and *H. eutropha* (124) and spinach (163). The K_i's were 8 to 15 mM. In contrast, the enzyme from *R. rubrum* was much more sensitive (K_i = 0.3 mM) and the opposite inhibition pattern was observed (9). The possibility that both citrate and phosphoglycerate inhibit the enzyme from *R. rubrum* by complexing Mg^{2+} should be re-evaluated in light of the possibility that this enzyme requires Mg^{2+}. In any case, the widely different sensitivities of RuDP carboxylases to these ligands may reflect two different reaction mechanisms. In this context, the failure of 1 mM 6-phospho-D-gluconate to inhibit the enzyme from *R. rubrum* in contrast to the striking inhibition of enzyme from *Hydrogenomonas* and spinach (39, 222) may also reflect different reaction mechanisms and active sites. As indicated in a previous section, it is likely that inhibition of RuDP carboxylase by phosphogluconate is of physiological importance.

Kuehn and McFadden examined the effect of a variety of metabolites upon the enzyme from *H. facilis* (G. D. Kuehn, Ph.D. thesis, Washington State University, Pullman, 1968). The following compounds tested at 1 mM in the presence of 20 mM HCO_3^-, 20 mM Mg^{2+}, and 1.6 mM RuDP, (124) gave the indicated level of inhibition: 5'-AMP, 42%; 2',3'-AMP, 44%; ADP, 42%; ATP, 34%; thiamine pyrophosphate, 33%; and L-glutamate, 35%. Because of the lack of specificity and reasonably high concentration of inhibitors required, the observed inhibitions may not have physiological significance. Under the same conditions, inhibition of 56% was observed by 1 mM fructose-1,6-diphosphate.

Quite recently Siegel and Lane (199) have extended earlier observations (243) which established that 2-carboxy-D-ribitol-1,5-diphosphate (CRDP) was a potent inhibitor of RuDP carboxylase. In the earlier studies, it was found that Mg^{2+} or Mn^{2+} promoted tight binding of CRDP to the enzyme. Fifty percent inhibition was obtained by 0.2 μM CRDP in the presence of 0.88 mM RuDP. Equimolar amounts of CRDP and metal ion were bound. The stoichiometry for RuDP binding was 8 mol per mol of enzyme at low ionic strengths in the presence of added metal ion, and the disassociation constant was 1 μM. Removal of tightly bound Cu^{2+}, known to be in the ratio of 1 g-atom/mol of enzyme (244), abolished the requirement for Mg^{2+} (or Mn^{2+}) in tight binding of CRDP. Because no significant catalytic or structural differences were found between the Cu^{2+}-saturated and Cu^{2+}-free enzyme (243), the role of Cu^{2+} remains obscure as does the unusual binding ratio with RuDP carboxylase. More recently, the formation of a carboxylase-Mg^{2+}-CRDP complex that undergoes a slow, temperature-dependent, conformational change has been inferred (199). The resultant state was inhibited and released CRDP extremely slowly. Of particular interest was the finding that ultraviolet difference spectra of RuDP carboxylase promoted by RuDP and CRDP were closely

similar, although Mg^{2+} was not present in studies with RuDP. These studies suggest that in the absence of metal ion RuDP can induce a conformational state not unlike that corresponding to the strongly inhibited state elicited by CRDP and Mg^{2+} at 30 C. Of interest, then, are data which suggest that preincubation of the enzyme with RuDP followed by addition of metal ion and HCO_3^- resulted in the lowest CO_2 fixation rate of all addition sequences tested (124, 138, 171). It seems likely that RuDP in the absence of Mg^{2+} and CO_2 can force the enzyme into a less active configuration which is similar to that formed with CRDP.

The basis of cyanide inhibition, which was shown to be uncompetitive with respect to RuDP for the spinach enzyme (242), was presumed at one time to be related to bound Cu^{2+} (244), although it has proven to be more complicated than this (243). Whatever the correct explanation, it is of some interest that RuDP carboxylases in extracts of *T. denitrificans*, *H. facilis* and *eutropha*, *Chromatium* D, *R. rubrum*, and *Chlorella pyrenoidosa* were all inhibited by 0.4 mM cyanide, a concentration which did not significantly deplete the concentration of RuDP by formation of the cyanhydrin (138).

There has been considerable speculation about the involvement of a sulfhydryl (SH) group in catalysis by RuDP carboxylase (see, for example, reference 107). Certainly reagents such as *p*-hydroxymercuribenzoate (PMB), *N*-ethylmaleimide, iodoacetate, and $HgCl_2$ rapidly inactivate the pure or highly purified enzyme from *Hydrogenomonas* (125; G. D. Kuehn, Ph.D. thesis, Washington State Univ., 1968), *Rps. spheroides* (3), soybean (26), wheat (211), and spinach (213). Of particular interest have been studies in Nagoya which established that inactivation of a molecule of spinach enzyme started only after modification of 10 of 96 SH groups by PMB and was complete after modification of 30 SH groups (213, 219). Use of ^{14}C-iodoacetamide established that four to five SH groups could be alkylated before onset of loss of activity and that complete loss was associated with the blocking of eight to ten SH groups (214). Preincubation of the enzyme with RuDP protected against inactivation by both reagents, but the addition of bicarbonate and Mg^{2+} resulted in inactivation by iodoacetamide that was somewhat faster than in the absence of RuDP (214). These results suggest that there are reactive SH groups in the higher plant enzyme that are not required for catalytic activity. A second class of somewhat less reactive SH groups is required for catalytic activity. Presumably, this class is exposed during catalytic turnover but is unavailable upon binding of RuDP in the absence of Mg^{2+} and HCO_3^-. These latter observations further suggest the presence of a configuration that is elicited by RuDP in the absence of HCO_3^- and Mg^{2+} but which is significantly different from the catalytically active species. Unfortunately, none of these experiments establishes whether SH groups function directly in catalysis or even whether they are at active sites.

Molecular size. The molecular weights and sedimentation coefficients of numerous RuDP carboxylases in varying states of purity have been estimated by several techniques. Although some must be considered as tentative because of enzyme impurity and inadequacy of techniques employed, the values, procedures used, and purity of the preparations are tabulated in Table 3.

One sedimentation coefficient of $S_{rel} = 17$ Svedberg units reported for impure enzyme from *T. thioparus* and *T. neapolitanus* (134) was omitted from Table 3 because that value is now believed to be at least 20% too high (R. D. MacElroy, personal communication). Data for the higher plant enzyme are restricted to those preparations that were pure or almost pure. For a somewhat more comprehensive tabulation of properties of the higher plant enzyme, the article by Kawashima and Wildman may be consulted (107). Among the higher plant enzymes, a seriously discrepant molecular weight (475,000) was obtained by Pon for the spinach enzyme of very low specific activity (170), although a larger species (mol wt 545,000) was also indicated. For those studies, approach to sedimentation equilibrium was used for times of up to 40 h. In light of the low specific activity and complex quaternary structure of the enzyme (next section), it seems likely that the preparations studied were heterogeneous initially or partially dissociated in the course of long-term sedimentation, or both.

The molecular weight of RuDP carboxylase from *Chlorella ellipsoidea* of 4.7×10^5 (Table 3) may be closer to 5.9×10^5 (215). There are a few inexplicable discrepancies in molecular weight for the carboxylase from a given organism (Table 3). The molecular weight is reported to be 83,000 and 120,000 for the enzyme from *R. rubrum* and 240,000 and 360,000 for the enzyme from *Rps. spheroides* by two different laboratories. Nevertheless, a clear-cut division of RuDP carboxylases into three size classes is possible: small (mol wt 80,000–120,000), intermediate (mol wt 240,000–360,000), and large (mol wt >500,000). In the latter category are pure

TABLE 3. *Molecular weights and sedimentation coefficients of RuDP carboxylases*

Source	State of enzyme purity	Sedimentation coefficient (Svedbergs)	Mol wt	Methods of measurement	Reference
Purple non-sulfur bacteria					
Rhodospirillum rubrum	Impure	$S_{20,w}$ = 6.2	1.2×10^5 [a]	Sedimentation, gel filtration	9
R. rubrum	Impure		8.3×10^4	Gel filtration	3
Rhodopseudomonas spheroides	Impure		2.4×10^5	Gel filtration	3
Rps. spheroides	Impure	S_{rel} = 14.5	3.6×10^5 [b]	Density gradient centrifugation	10
Rps. palustris	Impure	S_{rel} = 12	3.6×10^5 [b]	Density gradient centrifugation	10
Chemolithotrophic bacteria					
Thiobacillus denitrificans	Impure		3.5×10^5	Gel filtration	138
Hydrogenomonas facilis	Pure	$S_{20,w}$ = 20.0	5.5×10^5 [c]	Sedimentation equilibrium	125
H. eutropha	Pure	$S_{20,w}$ = 20.0	5.2×10^5 [c]	Sedimentation equilibrium	125
Purple sulfur bacteria					
Chromatium D	Impure	S_{rel} = 18	6.6×10^5 [b]	Density gradient centrifugation	10
Chromatium D	Impure	S_{rel} = 16–18		Density gradient centrifugation	114
Chromatium D	Pure?		5.5×10^5	Gel filtration	1
Blue-green algae					
Anacystis nidulans	Impure	S_{rel} = 19.5	6.6×10^5 [b]	Density gradient centrifugation	10
Plectonema boryanum	Impure	S_{rel} = 16–18		Density gradient centrifugation	114
Green algae					
Chlamydomonas reinhardi	Impure	S_{rel} = 16–18		Density gradient centrifugation	114
C. reinhardi	Pure		$5–5.6 \times 10^5$	Polyacrylamide gel electrophoresis	68
Euglena gracilis	Impure	S_{rel} = 19	6.6×10^5 [b]	Density gradient centrifugation	10
Chlorella ellipsoidea	Pure	$S_{20,w}$ = 19.0		Sedimentation	241
C. ellipsoidea	Impure	$S_{20,w}$ = 18.4	4.7×10^5	Sedimentation, gel filtration	2, 217
Higher plants					
Spinach	Pure?	$S_{20,w}$ = 18.3		Sedimentation	110
Spinach	Pure	$S^0_{20,w}$ = 21.0	5.6×10^5 [d]	Sedimentation equilibrium	163
Spinach	Pure	$S_{20,w}$ = 16.8		Sedimentation	241
Spinach-beet	Pure	$S^0_{20,w}$ = 18.3	5.6×10^5 [d]	Sedimentation, sedimentation equilibrium	186
Bean	Pure	$S_{20,w}$ = 16.5		Sedimentation	241
Wheat	Pure	$S_{20,w}$ = 18.5		Sedimentation	211
Chinese cabbage	Pure	$S_{20,w}$ = 17.0	5.1×10^5 [e]	Sedimentation equilibrium	114
Avena sativa	Pure?	$S^0_{20,w}$ = 18.2	5.7×10^5 [d]	Sedimentation, Stokes radius	208
Tobacco	Impure	$S_{20,w}$ = 18.3		Sedimentation	109

[a] Estimated from the Stokes radius determined by gel filtration with an assumed partial specific volume.

[b] Approximated from S_1/S_2 = (mol wt$_1$/mol wt$_2$)$^{2/3}$ using S_{rel} value and the molecular weight of *R. rubrum* RuDP carboxylase.

[c] Partial specific volume measured by sedimentation equilibrium in D_2O and H_2O (only for enzyme from *H. eutropha*) and estimated from amino acid composition.

[d] Partial specific volume assumed from amino acid composition.

[e] Partial specific volume assumed.

enzymes that have been less thoroughly characterized from *Ectothiorhodospira halophila* (B. A. McFadden and F. R. Tabita, unpublished data), an obligately halophilic member of the purple sulfur bacteria (*Thiorhodaceae*), and *Rumex*, maize, bean, and *Elodea* (70).

Quaternary structure, composition, terminal and tryptic peptides. Electron microscope examination of Chinese cabbage RuDP carboxylase first revealed complex quaternary structure in 1965 (75). More recent electron microscope investigations of the enzyme from other higher plants have been summarized (107), and electron micrographs of the *Chlorella* enzyme have been described (146). The results have established similarities between the plant enzymes but have not elucidated the number and precise arrangement of subunits.

In 1967 Rutner and Lane (192) and Sugiyama and Akazawa (211) dissociated the spinach and wheat RuDP carboxylases, respectively, with sodium dodecyl sulfate (SDS). The presence of two well-separated subunit types was revealed by polyacrylamide gel electrophoresis. Separation of the two subunit types by preparative chromatography on Sephadex in the presence of SDS enabled determination of the amino acid composition of each spinach subunit type (192). The reduced, carboxymethylated spinach beet enzyme was subsequently dissociated into two subunit types in 8 M urea, and the composition and molecular weights of each were determined (150). The molecular weights (by gel filtration) were 54,000 and 16,000, and those of the spinach subunits which were estimated by SDS-polyacrylamide gel electrophoresis were 55,800 and

12,000 (191). Thus it was of considerable interest that Kuehn and McFadden found only one gel electrophoretic component after dissociation of the *H. eutropha* enzyme with either urea or SDS, although dissociation was complex. The approximate molecular weight in either 3 to 6 M guanidinium chloride or 8 M urea was 40,700 and was 38,000 for the *H. facilis* enzyme in the presence of guanidinium chloride (125).

The amino acid compositions of the *Hydrogenomonas* enzymes (125) are compared with those for spinach subunit peaks (types) 1 and 2 (192) in Table 4. As is evident, the spinach subunits differ from one another especially in glycine, alanine, isoleucine, tyrosine, histidine, and arginine content. Marked differences between the composition of subunits 1 and 2 have also been noted for the spinach beet enzyme (150) and for the enzymes from *Chlorella ellipsoidea* (mol wt 58,000 and 15,000; reference 215) and five species of *Nicotiana* (mol wt 52,000 and 13,000; references 103, 108, 105). Comparisons reveal a striking similarity between compositions of subunit type 1 and disparity between compositions of subunit type 2 (107). It was of interest that the amino acid compositions of RuDP carboxylase from *Hydrogenomonas* resembled that of spinach subunit type 1 (Table 4), suggesting that catalytic potential would reside in that subunit.

TABLE 4. *Amino acid compositions of Hydrogenomonas RuDP carboxylase and spinach RuDP carboxylase subunits normalized (in mole ratios) with respect to phenylalanine*

Amino acid	Source			
	Hydrogenomonas facilis	*H. eutropha*	Spinach subunit 1	Spinach subunit 2
Aspartate	2.58	2.44	2.18	2.14
Threonine	1.25	1.51	1.75	1.19
Serine	1.00	1.16	0.81	0.77
Glutamate	2.08	2.20	2.20	2.26
Proline	1.08	1.29	1.13	1.56
Glycine	2.00	2.02	2.10	1.15
Alanine	2.08	2.44	2.16	0.86
Valine	1.58	1.64		
Isoleucine	1.08	1.02	0.88	0.56
Leucine	1.75	1.80	2.09	1.63
Tyrosine	0.69	0.82	0.92	1.57
Phenylalanine	(1.00)	(1.00)	(1.00)	(1.00)
Lysine	0.80	0.88	1.18	1.21
Histidine	0.56	0.56	0.67	0.45
Arginine	1.42	1.60	1.44	0.99
Half-cystine	0.58	0.69		
Methionine	0.68	0.76	0.42	0.46
Tryptophane	0.34	0.40		

Although this has not been rigorously established, it is noteworthy that most of the 8 to 10 SH groups associated with catalytic activity are found on the large subunit of the spinach enzyme (212, 216).

With regard to cysteine and cystine content, the half-cystine analyses of the enzyme from spinach beet (186), *Avena sativa* (208), and spinach (192) were similar, falling into the range of 0.4 to 0.5 when normalized with respect to the mole content of phenylalanine (107). On the basis of studies with PMB, it is likely that all of the half-cystine in the spinach enzyme is accounted for by readily titratable cysteine residues, i.e., there are no cystines (213). There are about 96 SH groups per molecule of spinach RuDP carboxylase and a similar number in the wheat enzyme (211, 213). In contrast, the enzymes from *Hydrogenomonas* have a similar half-cystine content in comparison with the higher plant enzymes (0.6–0.7, when normalized with respect to the mole content of phenylalanine), but less than one-third are cysteine residues on the basis of titrations with 5,5′-dithiobis(2-nitrobenzoate) in the presence of 8 M urea (125). Of these, more than 70% are buried and become reactive only in the presence of urea. These data suggest marked differences between the oxidation states of the higher plant and hydrogenomonad enzymes and, perhaps, differences in SH environment. Perhaps these disimilarities are related to the apparent differences in quaternary structure.

Recent studies of carboxy-terminal peptides have also established subunit heterogeneity for the plant enzyme (212, 215). The C-terminal residue was val for the large subunit (type 1) from spinach and the sequence was -ala$_4$-(val$_3$, ser$_2$)-leu for the large subunit from *Chlorella*. For the small subunit, sequences were -phe$_5$-leu$_4$-(tyr$_3$,thr$_2$)-tyr$_1$ and -ser$_2$-val$_1$ for spinach and *Chlorella* proteins, respectively. Other studies have suggested two types of subunits for RuDP carboxylase from *Chromatium* D (mol wt 57,000 and 12,000; reference 1), *Chlamydomonas reinhardi* (mol wt about 52,000 and 17,000; reference 68), and *Rumex* (mol wt 56,000 and 18,000; reference 70).

Recently, tryptic fingerprinting was undertaken with RuDP carboxylase from spinach and five species of *Nicotiana* (105, 110). Unfortunately, the enzyme from *N. tabacum*, although crystalline (109), revealed a second minor component during sedimentation (105, 106, 109) and gel electrophoretic analysis was not reported. Moreover, the specific activities of both the tobacco and spinach enzymes were extremely low (0.05 μmol of CO_2 fixed per min per mg of protein at 30 C; reference 110; cf 1.42 for

the spinach enzyme, reference 163), although that of the tobacco enzyme could be somewhat increased by storage at 25 C (106). Nevertheless, it is of interest that the tryptic fingerprints of subunit type 1 were reasonably similar for enzymes from all these sources whereas those for subunit type 2 were markedly different. The conclusion reached from comparison of the number of peptides observed with the lysine and arginine content, that the large subunit (mol wt 52,000; reference 108) derived from the *Nicotiana* enzyme was composed of two fused homologous peptides (105), must be re-evaluated in light of the recent finding that the number of peptides was almost twice (37) that found earlier.

Progress towards our understanding of the quaternary structure of RuDP carboxylase has been limited until recently by tedious purification procedures required to obtain pure enzyme. However, the technique of centrifugation of extracts into density gradients developed by Goldthwaite and Bogorad (70) promises access to one-step purification of the large enzyme. The procedure has been used successfully to obtain pure enzyme from *Hydrogenomonas*, *Ectothiorhodospira*, and *Chromatium* D (189, 222; B. A. McFadden and F. R. Tabita, unpublished data). Other procedures will have to be developed for purification of the bacterial enzymes of low and intermediate molecular weight.

To summarize, information suggests the occurrence of large (L) and highly variable small subunits (S) with molecular weights of 52,000 to 58,000 and 12,000 to 18,000, respectively, in RuDP carboxylase from green algae, higher plants, and *Chromatium* D. All of these enzymes have molecular weights that are greater than 500,000. The large and small subunits can be separated after dissociation by such agents as SDS, urea, or alkali, although use of the latter may result in complex dissociation (108). In general, the most likely stoichiometry of combination for these separable species is L_8S_8.

Nothing is known of the precise arrangement of the two subunits. The recent crystallization of the tobacco enzymes (36, 109, 126) may, however, open the way to a better comprehension of the quaternary structure. In marked contrast is RuDP carboxylase from *Hydrogenomonas* which is large but may be composed of a single type of subunit (mol wt $\cong$ 40,000).

Immunological relationships. Considerable research has been done on the immune response in rabbits elicited by RuDP carboxylase although not always with rigorously pure antigen (Table 5). Titrations of extracts with antibody to the enzyme from *H. eutropha* revealed a much closer relationship with *H. H 1* than with *H. H 16* in terms of RuDP carboxylase (138). This is of some interest because *H. H 1* and *H. H 16* were considered to be type strains of *H. eutropha* (46).

Other studies showed that some antigenic sites of RuDP carboxylase from *H. eutropha* were also shared with the enzyme from *H. facilis* and *R. rubrum* and, probably, from *Chromatium* D and *Chlorella pyrenoidosa*. Little if any cross-reaction was observed for the enzyme from *T. denitrificans* (138), nor did the anti-spinach RuDP carboxylase inhibit the enzyme from *Rps. spheroides* (3). A close resemblance has been found between the enzymes from *Chlorella ellipsoidea* and spinach (146) and between enzymes from spinach and tobacco (110). A slight precipitin was obtained in double diffusion studies of anti-spinach RuDP carboxylase against enzyme from *Chromatium* D, and a marked spur was observed with adjacent precipitin for the homologous reaction. Data with antibodies to the spinach and *N. tabacum* enzymes suggested that the enzymes shared one group of antigenic sites but that there was an additional group present on the tobacco but not the spinach enzyme (103).

These results suggest that more quantitative studies using complement fixation will be fruitful. For these, a judicious selection of antigenic RuDP carboxylases that span phylogenetic relationships between autotrophs should be made. Efforts should also be made to obtain immune responses to each of both subunits if they can be recovered in reasonably native states.

Origins of synthesis in eukaryota. Studies described in 1970 first indicated that the large and small subunits of RuDP carboxylases were synthesized on ribosomes in different cellular compartments. Kawashima (104) found that $^{14}CO_2$ supplied for 15 min to tobacco plants resulted in much higher specific radioactivity of large subunits than of small. The radioactivity was largely recovered in eight amino acids. Criddle et al. (45) established that chloramphenicol specifically inhibited the synthesis of the larger subunit of barley RuDP carboxylase. Cycloheximide exerted a primary effect upon synthesis of the smaller subunit and influenced production of the larger subunit by rapidly inhibiting total protein synthesis. Very recently, Ellis et al. (in Symposium on Nitrogen Metabolism in Plants, in press) established that pea chloroplast preparations containing 40 to 50% intact chloroplasts incorporated labeled amino acids into protein upon illumination. The incorporation was reduced by inhibitors of photophosphorylation and D-threo chloramphenicol and lincomycin. Actinomycin D, which inhib-

TABLE 5. *Immunological relationships of RuDP carboxylases*

Enzyme of	Antibody to enzyme from[a]				
	Hydrogenomonas eutropha[b]		Spinach		*Nicotiana tabacum*
	Double diffusion	Inhibition by (138)	Double diffusion	Inhibition by	Double diffusion
Hydrogenomonas eutropha (autotrophic)	Identity (138)				
H. eutropha (heterotrophic)	Identity	74%			
H. H 1	Identity	73%			
Rhodospirillum rubrum		53%		0 (2)	
H. H 16	Faint precipitin	40%			
Chlorella pyrenoidosa		30%			
H. facilis	No precipitin	30%			
Chromatium D	No precipitin	21%	Spur (1)		
Thiobacillus denitrificans	No precipitin	7%			
Rhodopseudomonas spheroides				0 (3)	
Chlorella ellipsoidea			Spur (1)	70% (146)	
Spinach			Identity (217)	50% (2)	Spur (110)
			Identity (110)	82% (146)	
Nicotiana tabacum			Spur (110)		Identity (110)

[a] References are given in parentheses.
[b] Antibody to pure enzyme from fructose-grown *H. eutropha* (138).

ited uridine incorporation into ribonucleic acid (RNA), had no effect upon protein synthesis. Of particular interest was the observation that the label had been incorporated into several membrane proteins and one soluble protein when products were examined by SDS-polyacrylamide gel electrophoresis after lysing the chloroplasts. The soluble protein, which was a major radioactive product, could not be sedimented by centrifugation at 150,000 × *g* for 1 h and ran exactly with the large subunit of RuDP carboxylase on SDS-gels polymerized from 8, 10, and 12% acrylamide.

When ^{35}S-methionine was used for incorporation studies, tryptic peptides derived from the soluble protein, which had a molecular weight of about 700,000, were identical to those derived from the large subunit of RuDP carboxylase which had been labeled in vivo (R. J. Ellis, personal communication). Separation of this labeled product from endogenous RuDP carboxylase would afford an unambiguous test for catalysis by an oligomer lacking the small subunit. These intriguing results suggest that the large subunit of RuDP carboxylase can be synthesized in the light on chloroplast ribosomes utilizing preformed messenger RNA which is coded for by chloroplast DNA.

Using another approach, Kawashima and Wildman have established the loci of DNA encoding the primary structure of the large and small subunits in higher plants. By using a unique peptide derived from the small subunit of RuDP carboxylase from *Nicotiana tabacum* as a phenotypic marker, these workers established that the marker was transmitted in a Mendelian manner in hybrids (111). In contrast, a unique peptide derived from the large subunit of another tobacco species was transmitted into hybrids by maternal inheritance (37).

In *Chlamydomonas reinhardi*, studies with inhibitors of protein synthesis and studies of amino acid incorporation into large and small subunits have been less conclusive (68, 145). Either both subunits are made on the same type of ribosome or, more plausibly, a very efficient coupling mechanism shuts off synthesis of the complementary subunit when the synthesis of a given subunit is inhibited (68).

In conclusion, the evidence establishes that nuclear DNA and chloroplast DNA code for the primary structures of the small and large subunits, respectively, of RuDP carboxylase in higher plants. Translation of the messenger RNAs probably occurs on cytoplasmic and chloroplast ribosomes, respectively. Of deep interest will be the mechanism by which the smaller subunit is transferred to the developing chloroplast to enable completion of the assembly of RuDP carboxylase.

CONCLUDING REMARKS

Echlin has discussed geochemical and paleological indications that the process of photosynthesis is an ancient one and that the first

photosynthetic organisms were on this planet some 3 billion years ago. Although ultrastructural details are lacking, these photosynthetic organisms were probably similar to modern photosynthetic bacteria in several ways. Within 300 million years, blue-green algae were prominent photosynthetic organisms, and it was not until between 1.5 and 1 billion years ago that the first eukaryotic photosynthetic organisms appeared (53). There is general agreement among botanists that green algae are the most likely ancestors of vascular plants because of the close biochemical similarities between the two groups. Moreover, the green algae are one of the few algal groups that have made the transition from marine to fresh water (54).

Echlin has discussed the ultrastructural characteristics of photosynthetic bacteria including blue-green algae and of the photosynthetic apparatus in eukaryotic cells (54). There are similarities found between membranous configurations in old cells of blue-green algae and the prolamellar body of greening eukaryotic chloroplasts (127). In comparison with membranous systems of non-O_2-evolving photolithotrophs, in general the lamellae in blue-green algae are in large sheets and there are no highly stacked arrays or tubular thylakoids (54). Polyhedral inclusion bodies, which are possibly analogous to those found in some chemolithotrophs (see following passage), have been observed in the blue-green algae (148). In examining the ultrastructure of other photosynthetic bacteria for clues to early evolution of autotrophism, the diversity is quite striking (54). Only the green sulfur bacteria (*Chlorobacteriaceae*) have structures in common, membrane-bound vesicles, that serve as the photosynthetic apparatus in members of *Chlorobium* (41, 42), purified *Pelodictyon chlathratiforme* (168), and *Chloropseudomonas ethylicum* (89), although there is some question as to the identity of the latter organism (72). In contrast, various *Thiorhodaceae* and *Athiorhodaceae* have membranous vesicles (24, 41, 47, 52, 60, 61, 83, 90, 236, 240) or lamellar systems (24, 43, 51, 52, 64–66, 84, 91, 182, 184, 226, 229, 235), and one member of *Thiorhodaceae*, *Thiococcus*, has tubular membranes (55). An increase in complexity from a supposedly primitive membrane system to the more "advanced" system of large peripheral vesicles found in the green bacteria has been postulated (91). Although this appears to be reasonable, hypotheses regarding present-day prototypes of primitive membrane systems appear to be premature because of the wide variation in ultrastructure of the *Thiorhodaceae* and *Athiorhodaceae* and the well-known influence of growth conditions, especially light intensity, upon the fine structure (54).

Because all chemolithotrophic bacteria are aerobic, the time of emergence of the first chemosynthetic organism depends critically upon the time of appearance of oxygen in the atmosphere. Were the oxygen of photosynthetic origin, most or all of these species would have been established after the blue-green algae about 2.8 billion years ago. Alternatively, they may be more ancient if oxygen was available as a result of ultraviolet photolysis of water. An examination of the ultrastructure of these bacteria reveals diversity. *Hydrogenomonas facilis* (82), *H. eutropha* (237), and *Micrococcus denitrificans* (116) have no unusual internal structure. Several studies of the thiobacilli revealed no unusual internal structure (144, 183, 231) aside from polyhedral inclusion bodies seen in *T. ferroxidans* (239), *T. thiooxidans* (144), and a marine thiobacillus (224). However, a recent detailed comparative study revealed that these bodies were also present in *T. thioparus*, *T. neapolitanus*, and *T. intermedius*. In addition, lamellar bodies similar to those in some photosynthetic bacteria were occasionally found in *T. thioparus*, and a few cells of *T. intermedius* had paracrystalline bodies (198). In contrast, *Nitrocystis oceanus*, a marine nitrifier (153), *Nitrosomonas europaea* (153), *Nitrobacter agilis* (153, 173), and *N. winogradskyi* (232) have highly lamellar internal structures. *N. agilis* contains polyhedral inclusion bodies (153, 173), and there may be corresponding structures in *N. winogradskyi* (232). *Methanobacterium thermoautrophicum* also has a lamellar internal membrane structure (249), as do the methane-utilizing bacteria, which either have stacked membranes concentrated around the periphery or bundles of disk-shaped membrane vesicles distributed throughout the cell (176). In recapitulation, all obligately chemolithotrophic genera examined have some members, at least, that possess a lamellar internal structure, which is also found in some photosynthetic bacteria, but it is difficult to discern any clear-cut relationships or ultrastructural trends. Of special interest may be polyhedral inclusion bodies of unknown function that are frequently observed in species of *Thiobacillus* and *Nitrobacter*. Clearly, more research is needed on the relationship of structure and function and the variation of fine structure with growth conditions.

Energy-trapping mechanisms in autotrophic species are quite diverse. Among photolithotrophic bacteria which do not evolve oxygen a single photosystem is utilized, whereas O_2-evolving species including blue-green algae function with two photosystems. Olson has

speculated about evolution of the second photosystem (157). Chemolithotrophs derive energy from the oxidation of numerous inorganic substances such as Fe^{2+}, H_2, NO_2^-, NH_3, $S_2O_3^{2-}$, and S^{2-}. Unfortunately, information about the molecular details of these oxidations is insufficient to provide a circumspect postulate about evolutionary sequence. One important property, however, is shared by all autotrophic species, and that is the assimilation of carbon dioxide via the Calvin cycle. The acquisition of two catalysts in this cycle, RuDP carboxylase and phosphoribulokinase, may have triggered (or corresponded with) the emergence of autotrophic life. In concluding, I shall summarize the properties of RuDP carboxylase in the context of evolution.

The molecular weights of RuDP carboxylase fall into three size categories: small (mol wt ~100,000), intermediate (mol wt ~300,000), and large (mol wt ~500,000).

If increasing molecular weight of this enzyme is a consequence of later evolutionary development, the following sequence of evolution of photosynthetic forms can be inferred: *Rhodospirillum* (*Athiorhodaceae*) → *Rhodopseudo-* (*Athiorhodaceae*) → *Chromatium* and *Ectothiorhodospira* (*Thiorhodaceae*) and O_2-evolving species (blue-green algae, green algae, higher plants).

Consistent with this trend is the observation that only the large enzymes are sensitive to inhibition by 6-phosphogluconate. Presumably, the green sulfur bacteria (*Chlorobacteriaceae*) are more advanced than the *Thiorhodaceae* as reflected by their internal structure and deeper commitment to autotrophy. It will be of interest to compare RuDP carboxylase from the green sulfur bacteria with that of *Chromatium*, which is known to have quaternary structure closely similar to that for the enzyme from green algae and higher plants (two subunit types of mol wt ≅55,000 and 15,000). It will also be of interest to examine the quaternary structure of the enzyme from blue-green algae and to assess quantitatively the immunological relationships of the enzyme and subunits from selected autotrophic forms. Comparisons of tryptic fingerprints of subunits and, eventually, of their primary structures will be feasible and interesting. Scrutiny of the natural distribution of phosphoglycolate production by RuDP carboxylases will also be important.

With regard to evolution of the chloroplast, if one favors an exogenous origin the most logical precursor was a blue-green alga that became endosymbiotic with a nonphotosynthetic organism. Ultrastructural and biochemical information as well as examples of possibly analogous endosymbiosis have been reviewed (54) and lend considerable credence to this theory of chloroplast origin. Of special interest in this connection will be the quaternary structure of the blue-green algal RuDP carboxylase. If it contains large and small subunits, which in higher plants, at least, are encoded within the chloroplast and nucleus, respectively, it would suggest that part of the genome of the progenitor of the chloroplast, i.e., the blue-green alga, was integrated into the DNA of the endosymbiotic partner.

Positioning chemolithotrophic bacteria among evolving autotrophs remains an enigma but only because there is a paucity of data on RuDP carboxylase from these forms. Only the enzyme from *Hydrogenomonas* has been thoroughly characterized in terms of molecular properties. It is composed of about 12 closely similar if not identical subunits (mol wt ≅40,000). In this respect it differs markedly from the *Chromatium* and plant enzymes yet responds similarly to 6-phosphogluconate, as does the enzyme from blue-green algae. On the other hand, RuDP carboxylase from *T. denitrificans* is of intermediate size and is insensitive to 6-phosphogluconate (222). In this respect, it is closely similar to the enzymes from *Athiorhodaceae* and it may be significant that *T. denitrificans* is unusual among chemolithotrophic bacteria in that it may be cultured anaerobically or aerobically. Of obvious significance will be comparisons of RuDP carboxylase from the chemosynthetic bacteria including organisms such as *Micrococcus denitrificans* and *Methanobacterium thermoautotrophicus*, if the enzyme is found in the latter organism. Also of interest will be a search for a possibly analogous enzyme among the methane-utilizing bacteria.

In the final analysis, it is evident that knowledge of the structure, function and regulation of RuDP carboxylase will yield much information about the evolution of autotrophism.

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ADDENDUM

After this article was completed, three relevant publications on RuDP carboxylase appeared. Two described the oxygenase activity (T. J. Andrew, G. H. Lorimer, and N. E. Tolbert. 1973. Ribulose diphosphate oxygenase. I. Synthesis of phosphoglycolate by fraction-I protein of leaves. Biochemistry **12**:11-18, and G. H. Lorimer, T. J. Andrews, and N. E. Tolbert. 1973. Ribulose diphosphate oxygenase. II. Further proof of reaction products and mechanism of action. Biochemistry **12**:18-23) and the third described the six-carbon reaction intermediate (B. Sjödin, and A. Vestermark. 1973. The enzymatic formation of a compound with the expected properties of carboxylated ribulose 1,5-diphosphate. Biochim. Biophys. Acta **297**:165-173). Also, my attention was drawn to a study of the effect of energy charge upon phosphoribulokinase (B. A. Hart, and J. Gibson. 1971. Ribulose-5-phosphate kinase from *Chromatium* sp. strain D. Arch. Biochem. Biophys. **144**:308-321).

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