Role of Aldolase in Photosynthesis. I. Enzyme Studies With Photosynthetic Organisms With Special Reference to Blue-Green Algae [1,2]

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Hexose formation during photosynthesis has been presumed to occur by means of the reductive pentose phosphate cycle (3). An integral part of this cycle is the condensation of two molecules of triosephosphate to yield fructose-1,6-diphosphate (FDP) in a reaction catalyzed by aldolase. In support of aldolase participating in photosynthesis, Tewfik and Stumpf (35) showed that aldolase was present in photosynthetic tissues of all the higher plants which they examined. There is, however, evidence that this enzyme is absent or very low in some photosynthetic organisms. Jacobi (11) noted the absence of aldolase in four species of red algae. Szymona and Doudoroff (33) could find only traces of the enzyme in *Rhodopseudomonas spheroides*. Richter (24,25) has reported that the enzyme cannot be detected in the blue-green alga, *Anacystis nidulans*, or in a strain of *Rhodopseudomonas spheroides*. Peterkofsky and Racker (20) have recently determined the activities of several enzymes of the reductive pentose phosphate cycle in spinach, Euglena, and in a high temperature and a low temperature strain of Chlorella. Their data showed that in all four organisms transaldolase, sedoheptulose-1,7-diphosphatase and fructose-1,6-diphosphatase were present in insufficient amounts to support the rate of CO_2 fixation by intact cells. Furthermore, aldolase was also low in both Euglena and the low temperature strain of Chlorella.

The present study is an attempt to assess the role of aldolase in photosynthesis. In the first paper of this series, aldolase and enzymes associated with the reductive pentose phosphate cycle have been determined in various organisms, with special reference to the Cyanophyceae algae.

Materials & Methods

▶ Materials. FDP was prepared from a commercial sample obtained from the Nutritional Biochemical Corp. Calcium-FDP was converted to the strychnine salt as described by Mandl and Neuberg (17) and after crystallization from 85 % ethanol, the sodium salt was prepared by the method of Sable (27). The purified salt contained no detectable inorganic phosphate (Pi).

The barium salt of DL-glyceraldehyde-β-P diethylacetal was obtained from the Sigma Chemical Co. and cyclohexylammonium dihydroxyacetone-P dimethylketal from the California Corporation for Biochemical Research. Glucose-6-P dehydrogenase was obtained from the Sigma Chemical Co. and 3-phosphoglycerate kinase and α-glycerolphosphate dehydrogenase from C. F. Boehringer and Soehne. A sample of crystalline rabbit muscle aldolase was a gift from Dr. E. Racker. Other materials were commercial preparations.

▶ Organisms. The green algae, *Chlorella pyrenoidosa* Chick. and *Scenedesmus obliquus* (Turp.) Kütz., were grown in a medium containing: (g/liter) KNO_3, 0.506; K_2HPO_4, 0.087; KH_2PO_4, 0.068; $MgSO_4 \cdot 7H_2O$, 0.493; $Ca(NO_3)_2$, 0.041; NaCl, 0.010; 1 ml iron solution (50 mg $FeSO_4 \cdot 7H_2O$, 20 mg tartaric acid/10 ml water) and 1 ml microelements (g/liter: H_3BO_3, 1.43; $MnSO_4 \cdot 4H_2O$, 1.05; $ZnCl_2$, 0.05; $CuSO_4 \cdot 5H_2O$, 0.04, $Na_2MoO_4 \cdot H_2O$, 0.01). The cells were harvested after four to six days at 23 C under continuous illumination. The cultures were shaken continuously in a controlled-temperature water bath and aerated with a 5 % CO_2/95 % air mixture.

The blue-green algae, *Anacystis nidulans* (Richt.) Drouet and Daily, *Anabaena variabilis* Kütz. and *Nostoc muscorum* Kütz, (strains A & G) were obtained from Dr. J. Myers and grown in Medium C described by Kratz and Myers (15). Cell yields of 0.5 g wet weight/liter were obtained after 2 days at about 30 C under continuous illumination and aeration with 0.5 % CO_2/99.5 % air. *Tolypothrix tenuis* Kütz., obtained from Dr. A. Watanabe, was grown autotrophically in the light or heterotrophically with glucose in the dark as described by Kiyohara et al. (13). Air was dispersed through the solution and the cells harvested after 4 days at 32 C.

Chondrus crispus (L.) Stackhouse, a red alga, was obtained from Woods Hole Experiment Station. A culture of the red alga, *Porphyridium cruentum* (Smith & Sowerby) Näg., was provided by Dr. L. Provasoli and grown in the medium used by Pintners and Provasoli (21) to grow Phormidium. Sufficient

cells for harvesting were obtained after growth at about 30 C for 10 days without aeration.

The golden-brown alga, *Ochromonas malhamensis* Pringsheim, was grown in a skim-milk medium described by Reazin (23) under continuous illumination.

Rhodospirillum rubrum (von Esmarch) Molisch, was obtained from Dr. H. Gest and grown according to methods used in his laboratory. The medium used contained in g/liter: $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.075; $FeSO_4 \cdot 7H_2O$, 0.012; KH_2PO_4, 0.6; $K_2H\ PO_4$, 0.9; ethylenediamine tetraacetic acid, 0.02; $(NH_4)_2\ SO_4$, 0.5 with 15 μg biotin/liter, and 1 ml of a trace metal supplement (g/liter, $MnSO_4 \cdot 4H_2O$, 2.1; H_3BO_3, 2.8; $Co(NO_3)_2 \cdot 3H_2O$, 0.04; $ZnSO_4 \cdot 7H_2O$, 0.24; $Na_2MoO_4 \cdot 2H_2O$, 0.75). The glucose medium also contained 10 g/liter glucose and the malate medium 6 g/liter D,L-malic acid. All media were adjusted to pH 6.8 and the cells grown under continuous illumination at 25 C. 5 % CO_2/95 % H_2 was bubbled through the cultures and the cells harvested after 10 days.

Escherichia coli (Migula) Castellani and Chalmers, Crookes strain, was grown as previously described (18).

Cells were harvested by centrifugation and washed with 0.85 % NaCl. Enzyme activities were determined in extracts prepared with a Mickle disintegrator (green, blue-green, & golden-brown algae), a 9 KC Raytheon magnetostrictor oscillator (*R. rubrum*) or by grinding with sand or with levigated alumina with a pestle and mortar (red algae, spinach, & *E. coli*). Whole cells and debris were removed by low-speed centrifugation.

► *Enzyme assays.* Aldolase was determined by the following procedures. Method I. The routine assay for aldolase activity was that of Sibley and Lehninger (28) as modified by Bard and Gunsalus (2). The triose phosphates formed by the cleavage of FDP were measured colorimetrically as the 2,4-dinitrophenylhydrazine derivatives after using hydrazine as the trapping agent. Method II. The alkali-labile phosphate of the triose phosphates was used to follow aldolase activity after trapping the trioses with cyanide. The reaction mixture was that of Peanasky and Lardy (19) and phosphate was determined by the method of Taussky and Shorr (34). Method III. Aldolase activity was linked to DPNH oxidation with α-glycerolphosphate dehydrogenase essentially as described by Wu and Racker (36). The reaction mixture contained 90 μmole tris (pH 7.5), 10 μmole cysteine HCl (adjusted to pH 7.5 before addition), 0.3 μmole DPNH, 0.01 ml α-glycerolphosphate dehydrogenase, 5 μmole FDP and extract to be assayed in a total volume of 3.0 ml. Method IV. The condensation of glyceraldehyde-3-P and dihydroxyacetone-P was measured in a reaction mixture containing 30 μmole glycylglycine buffer (0.5 M solution adjusted to pH 7.5 with N NaOH), 2 μmole dihydroxyacetone-P, 2 μmole of DL-glyceraldehyde-3-P and extract to be assayed in a total volume of 2.0 ml. The rate of dis-

appearance of alkali-labile phosphate was followed as in Method II.

Fructose 1,6-diphosphatase activity was determined by measuring the rate of release of Pi during the hydrolysis of FDP in the following reaction mixture: 100 μmole buffer, 5 μmole FDP, 5 μmole $MgCl_2$ and enzyme extract in a volume of 1.5 ml. After 10 minutes at room temperature, the solution was deproteinized with 0.3 ml 20 % trichloroacetic acid and Pi measured. The acid diphosphatase was determined at pH 5.8 (acetate buffer) and the alkaline diphosphatase was assayed at pH 9.0 (glycylglycine buffer).

Phosphohexokinase and glucose-6-P isomerase activities were determined by the procedures of Axelrod et al. (1) and Wu and Racker (36), respectively. Glucose-6-P dehydrogenase was assayed by the method of Kornberg and Horecker (14) and gluconate-6-P dehydrogenase by the method of Horecker and Smyrniotis (10). Hexokinase activity was measured by the spectrophotometric precedure of Slein et al. (29). DPN-and TPN-linked glyceraldehyde-3-P dehydrogenases were assayed as the back reactions in systems containing 3-phosphoglycerate kinase as described by Wu and Racker (36). Phosphohexokinase and glyceraldehyde-3-P dehydrogenase activities were measured in extracts which had been prepared in the presence of 2×10^{-3} M cysteine HCl. Protein was determined with the Folin-Ciocalteu reagent (16) using bovine serum albumen as the standard.

Results

► Aldolase in Anacystis nidulans: In confirmation of Richter's results (24, 25), no FDP-aldolase activity could be detected in *A. nidulans* under any of the conditions used (table I). The four assay procedures gave uniformly negative results although experiments, made at the same time, showed consistent and reproducible activities in samples of Chlorella, spinach or a crystalline preparation from muscle. Attempts

Table I

Comparison of Aldolase Activities in Extracts of Various Photosynthetic Organisms*

Organism	Activity μmoles FDP split/mg protein/hr at 37 C
Anacystis nidulans	0
Anabaena variabilis	0
Nostoc muscorum, A & G	0
Tolypothrix tenuis	0
Chlorella pyrenoidosa	12.4
Scenedesmus obliquus	8.1
Chondrus crispus	7.0
Porphyridium cruentum	3.6
Ochromonas malhamensis	9.3
Rhodospirillum rubrum	2.3
Spinach	17.3

* Enzyme activities were determined with Method 1 as described in Materials & Methods.

to show activity with fructose-1-P, fructose-6-P or commercial FDP in the presence of *A. nidulans* extracts were also unsuccessful. Since the apparent pH optimum for aldolase activity has been found to vary with the method of assay (26) control experiments were made at pH values ranging from 6.5 to 9.5 but were all negative. Assays were also done at temperatures from 25 to 38 C and again yielded negative results.

The following procedures were also unsuccessfully used to try to demonstrate aldolase activity in *A. nidulans*: A. Cell extracts were prepared, both at 4 C and at room temperature, by means of the Mickle disintegrator, grinding with a pestle and mortar or by the preparation and extraction of acetone powders. Extracts were made in the presence and absence of 2×10^{-3} M cysteine HCl. Homogenates prepared with a pestle and mortar were also suspended in 20 % ethylene glycol or 20 % Carbowax 4000. It was not possible to prepare Mickle extracts in the presence of these compounds because of their high viscosity; B. Particles and supernatant solutions were prepared by centrifuging the cell homogenates at $4,000 \times g$, $10,000 \times g$, and $144,000 \times g$. The particles were also disrupted with 5 % Tween-80 or with 0.2 % digitonin. Although both Stocking (31) and Heber (9) have shown that aldolase activity can be unmasked or increased by differential centrifugation of extracts of some higher plants, no activity could be demonstrated with Anacystis; C. Ammonium sulfate precipitation was carried out at pH 4.5, 5.5, and 7.0 but no activity was detected in any of the fractions either immediately after preparation or after overnight dialysis against acetate buffer (pH 5.5), tris buffer (pH 7.4) or distilled water; D. A metal requirement for aldolase activity has been demonstrated in several organisms (30) but the addition of Mg^{++}, Mn^{++}, Fe^{++}, Zn^{++}, Co^{++}, or Ni^{++} at concentrations from 10^{-2} to 10^{-4} M failed to indicate activity in Anacystis; E. Kenton (12) described the activation of a latent phenolase from *Vicia faba* by anionic wetting agents but a similar treatment of extracts of Anacystis with sodium dioctylsulfosuccinate (Aerosol OT) or hexadecyltrimethylammonium bromide failed to reveal any aldolase activity: F. The addition of an extract of Anacystis did not affect the aldolase activity of Chlorella, spinach, or a crystalline preparation from muscle.

► Distribution of Aldolase in Other Organisms. Aldolase activity was determined in a number of organisms from various taxonomic groups and some of the results are given in table I. Three other blue-green algae also showed no detectable activity. *Nostoc muscorum*, strain A, which, unlike Anacystis, Anabaena, and *N. muscorum*, strain G, is said to show increased growth in the presence of glucose (15) was also inactive after growth in a medium containing 1 % glucose. *Tolypothrix tenuis* is unusual among blue-green algae in that it can be grown in the dark in a glucose medium. Again, however, both the photo-autotrophically and heterotrophically grown cells were devoid of activity. In contrast to this, all other photosynthetic organisms assayed showed aldolase activity; namely, green, red, and golden-brown algae, spinach, and Rhodospirillum. The red bacterium showed similar activities when grown in the basal, glucose, or malate media (2.3, 3.1, & 3.8 μmole FDP split/mg protein/hr, respectively). The activities in table I compare with 29.6 μmole/mg protein/hr for *E. coli*. It is noteworthy that aldolase activity in the red alga *Chondrus crispus* may be metal-dependent since 10^{-3} M KCN caused an 80 % inhibition and 10^{-3} M EDTA a 60 % inhibition. Neither Chlorella, Scenedesmus, nor spinach showed this effect and Stumpf (32) had previously demonstrated that the enzyme partially purified from pea seeds was unaffected by metal chelating agents.

Using the precedure described by Dische and Landsberg (6) for muscle aldolase, extracts of Chlorella and *E. coli* could be shown to carry out a condensation of formaldehyde and dihydroxyacetone-P. No reaction could be observed in the presence of Anacystis extracts.

► Survey of Other Enzyme Activities in Algae. Several enzymes associated with carbohydrate metabolism were assayed in spinach, blue-green, green,

Table II

Enzyme Activities in Extracts of Algae & Spinach*

Enzyme	Activity μmole/mg protein/hr at 25 C						
	Anacystis	Anabaena	Nostoc	Chondrus	Chlorella	Scene-desmus	Spinach
Alkaline fructose-1,6-diphosphatase	0.8	2.1	1.5	3.6	5.7	9.7	12
Acid fructose-1,6-diphosphatase	4.4	7.5	5.6	4.8	4.4	4.8	11
Phosphohexokinase	0.8	1.0	1.5	...	3.9	2.8	...
Glucose-6-P dehydrogenase	11	11	12	0.4	2.2	...	4.0
Gluconate-6-P dehydrogenase	31	17	21	0.8	2.5	2.6	3.2
Hexokinase	15	...	17	13	19	...	...
Glucose-6-P isomerase	36	...	24	16	21	...	...
Glyceraldehyde-3-P dehydrogenase, DPN	2.6	...	3.0	1.8	3.7	...	...
Glyceraldehyde-3-P dehydrogenase, TPN	4.8	...	2.8	1.0	8.4	...	...

* Enzyme activities of cell-homogenates were assayed as described in Materials & Methods.

and red algae, and the results are summarized in table II. Glucose-6-P and gluconate-6-P dehydrogenase were very active in all the blue-green algae, showed medium activity in Chlorella, Scenedesmus, and spinach and were virtually inactive in Chondrus. High levels of hexokinase and glucose-6-P isomerase could be detected in all organisms tested as could both DPN and TPN dependent glyceraldehyde-3-P dehydrogenases. Phosphohexokinase could be detected in all organisms tested but was less active in the blue-green algae. No phosphohexokinase activity could be detected unless the extracts were made in the presence of 2×10^{-3} M cysteine HCl; a similar phenomenon was noted with the glyceraldehyde-3-P dehydrogenases. The phosphatase activities showed marked differences between the groups of organisms. The green algae had relatively high fructose-1,6-diphosphatase activity at both low (5.8) and high (9.0) pH, whereas in the blue-green algae the acid fructose-1,6-diphosphatase was three to five times more active than the alkaline fructose-1,6-diphosphatase (table II).

Discussion

Although FDP aldolase is generally considered to be required for photosynthesis, the enzyme is apparently absent from the blue-green algae *Anacystis nidulans, Anabaena variabilis, Nostoc muscorum* (strains A & G), and *Tolypothrix tenuis* (table I). Since the methods of measuring this enzyme are known to have limitations (26), four separate assays were used in experiments with Anacystis. These methods gave reproducible and positive results with other organisms so that it is unlikely that the failure to demonstrate aldolase activity in the blue-green algae arose from difficulties in the assay procedure. It is possible that failure to demonstrate aldolase activity in vitro could be due to extreme lability or unusual properties of the enzyme from blue-greens; this seems unlikely in view of the large number of methods which were used to try to detect it and the known stability of aldolase from other organisms. Furthermore, no inhibitor could be detected. In addition, a number of other enzyme activities were readily detected in extracts of these organisms. These data, together with those of Richter (24, 25), would indicate that a lack of aldolase is characteristic of the Cyanophyceae (table I).

In contrast to the findings of Jacobi with four species of red algae (11), aldolase, hexokinase, and glyceraldehyde-3-P dehydrogenase were all demonstrated in the red alga *Chondrus crispus*. Aldolase was also detected in another red alga, *Porphyridium cruentum* (table I). The distribution of aldolase among the photosynthetic bacteria appears to be variable since, although the enzyme is barely detectable or even absent, in *Rhodopseudomonas spheroides* (25, 33), we found appreciable activity in *Rhodospirillum rubrum* and Fuller et al. (8) found aldolase in Chromatium.

The pattern of enzyme distribution in the blue-green algae differed from that in Chlorella, Scenedesmus, Chondrus, and spinach (table II). While acid diphosphatase values were essentially similar for all the organisms tested, alkaline diphosphatase, which is apparently specific for FDP (22), was considerably lower in the blue-greens than in the green algae and spinach. This may be because its substrate, FDP, is not formed in the absence of aldolase. Another enzyme involved in FDP metabolism, phosphohexokinase, was twice as active in the green algae as in the blue-greens. In confirmation and extension of Richter's observations with Anacystis (24), glucose-6-P dehydrogenase, and gluconate-6-P dehydrogenase were especially active not only in Anacystis but also in Anabaena and Nostoc. This suggests that the pentose phosphate pathway is the main pathway for hexose degradation in the blue-greens. The situation in the Cyanophyceae would seem to be analogous to the heterofermentative lactic acid bacteria (5) and the Pseudomonads (7) which do not have a glycolytic pathway and are also apparently devoid of aldolase. The presence of hexokinase in the blue-greens suggests that their inability to respire exogenous glucose in contrast to the green algae (15) is the result of a permeability problem.

While glucose-6-P dehydrogenase activity was very low in extracts of Chondrus, an extremely active glucose oxidase was detected (unpublished observation of this laboratory). This oxidase appears to be similar to the enzyme purified from another marine red alga, *Iridophycus flaccidum,* by Bean and Hassid (4): for instance, the rate of galactose oxidation was 93 % of the rate of glucose oxidation, which corresponds closely to the value obtained by these workers. These data suggest that the red algae can respire glucose and galactose by a pathway whose initial step(s) do not involve phosphorylated intermediates, as well as by the glycolytic pathway.

The data reported in this paper, in conjunction with results obtained by other workers, indicate that the pathways of glucose dissimilation are markedly different among the various groups of algae. The apparent absence of aldolase from four species of blue-green algae and from some photosynthetic bacteria indicates that these organisms may not assimilate CO_2 through 3-phosphoglycerate and FDP.

Summary

Several enzymes involved in carbohydrate metabolism were assayed in extracts of blue-green, green, red, and golden-brown algae, the photosynthetic bacterium *Rhodospirillum rubrum* (von Esmarch) Molisch, and spinach. Aldolase was absent from the blue-greens but could be detected in all the other organisms. Glucose-6-phosphate and gluconate-6-phosphate dehydrogenases were especially active in the three blue-greens examined and it is suggested that the pentose phosphate pathway is the main method of hexose dissimilation in these organisms. These dehydrogenases were low in the red alga *Chondrus crispus* (L.) Stackhouse, but a non-specific glucose

oxidase was very active. It is suggested that the apparent absence of aldolase from some photo-autotrophic organisms makes it likely that the formation of hexoses during photosynthesis does not necessarily proceed by the condensation of two molecules of triose phosphate.

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