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Biochemical Basis of Obligate Autotrophy in Blue-Green Algae and Thiobacilli

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Differential rates of incorporation of sugars, organic acids, and amino acids during autotrophic growth of several blue-green algae and thiobacilli have been determined. In obligate autotrophs (both blue-green algae and thiobacilli), exogenously furnished organic compounds make a very small contribution to cellular carbon; acetate, the most readily incorporated compound of those studied, contributes about 10% of newly synthesized cellular carbon. In *Thiobacillus intermedius*, a facultative chemoautotroph, acetate contributes over 40% of newly synthesized cellular carbon, and succinate and glutamate almost 90%. In the obligate autotrophs, carbon from pyruvate, acetate, and glutamate is incorporated into restricted groups of cellular amino acids, and the patterns of incorporation in all five organisms are essentially identical. These patterns suggest that the tricarboxylic acid cycle is blocked at the level of α-ketoglutarate oxidation. Enzymatic analyses confirmed the absence of α-ketoglutarate dehydrogenase in the obligate autotrophs, and also revealed that they lacked reduced nicotinamide adenine dinucleotide oxidase, and had extremely low levels of malic and succinic dehydrogenase. These enzymatic deficiencies were not manifested by the two facultative chemoautotrophs examined. On the basis of the data obtained, an interpretation of obligate autotrophy in both physiological and evolutionary terms has been developed.

Autotrophy, the ability to grow in a completely inorganic environment, occurs in two major physiological groups: phototrophs, and organisms that use reduced inorganic compounds as energy sources. In each major group, autotrophy may be facultative or obligate (Table 1). The facultative autotroph can meet its requirements for both carbon and energy by the use of organic compounds; the obligate autotroph cannot, and is always dependent on carbon dioxide as principal carbon source, and either light or a reduced inorganic compound as energy source.

Winogradsky, who first rigorously demonstrated the existence of obligate autotrophs through his studies on the nitrifying bacteria (46, 47), emphasized that organic compounds are not merely unutilizable by these organisms, but are inhibitory at relatively low concentrations (48). However, subsequent work has shown that obligate autotrophs are not, in general, unusually susceptible to growth inhibition by organic compounds. As early as 1902, Nathansohn (33) observed that *Thiobacillus thioparus* can grow in the presence of some simple organic compounds at concentrations as high as 0.5% (w/v). Later work on nitrifying bacteria (20, 24) demonstrated that relatively high concentrations of several organic compounds are not inhibitory, and may even stimulate growth. Similar observations have been made with blue-green algae (1, 22) and green bacteria (26, 38).

During the past 20 years, the use of tracer methods has shown that some organic compounds can be absorbed by obligate autotrophs and incorporated into cell material. The assimilation of one or more organic compounds has been demonstrated for thiobacilli (8; Kelly, Proc. Soc. Gen. Microbiol., p. v, 1965), *Nitrobacter* (12, 19), *Nitrosomonas* (9, 10), *Ferrobacillus* (C. C. Remsen and D. G. Lundgren, Bacteriol. Proc., p. 33, 1963), green bacteria (38), purple sulfur bacteria (36), and blue-green algae (4, 17). These findings exclude one possible physiological interpretation of obligate autotrophy, namely, that it reflects general impermeability of the cell to organic compounds (44). The only alternative interpretation is that obligate

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TABLE 1. *Distribution of obligate and facultative autotrophy among procaryotic organisms*

Organism	Type of autotrophy		Reference
	Obligate	Facultative	
Photoautotrophs			
Blue-green algae	+	+	1, 22, 25
Green bacteria			
Chlorobium	+	−	26
Chloropseudomonas	−	+	39
Purple sulfur bacteria			
Chromatium	+	+	36
Thiospirillum	+	−	40
Chemoautotrophs			
Nitrosomonas	+	−	46
Nitrosocystis	+	−	45
Nitrobacter	+	−	46
Thiobacillus	+	+	33, 41, 42
Ferrobacillus	+	+?	27, Remsen and Lundgren[a]
Hydrogenomonas	−	+	16

[a] Bacteriol. Proc., p. 33, 1963.

autotrophs have defects in the central pathways of intermediary metabolism which prevent the use of any organic compound as a general source of cell carbon and of energy. The experiments reported here were undertaken to determine as precisely as possible the nature of these metabolic defects. Three obligately photoautotrophic blue-green algae and two obligately chemoautotrophic *Thiobacillus* spp. served as the principal experimental objects. Two facultative chemoautotrophs, *Hydrogenomonas eutropha* and *Thiobacillus intermedius*, were also examined. These two bacteria represent slightly different variations on the theme of facultative autotrophy. Like all other hydrogen bacteria, *H. eutropha* can grow rapidly at the expense of many single organic compounds. *T. intermedius* cannot do so; heterotrophic growth occurs only in complex organic media, and its rate is accelerated by the addition of thiosulfate, the inorganic energy source (30).

MATERIALS AND METHODS

Biological material. Pure cultures of *Anacystis nidulans* (strain of Kratz and Allen, originally received from Mary Belle Allen), *Coccochloris peniocystis*, and *Gloeocapsa alpicola* (both originally received from G. P. Fitzgerald) were kindly provided by Mary Mennes Allen. *A. nidulans* and *C. peniocystis* are small, rod-shaped unicellular blue-green algae; *G. alpicola* is a coccoid unicellular blue-green alga. All appear to be obligate photoautotrophs, since attempts to obtain growth in the dark with many different organic compounds have consistently failed (M. M. Allen, *personal communication*). Stock cultures were maintained in flasks of liquid mineral medium incubated in a light cabinet at 28 C.

Strains of obligately autotrophic thiobacilli which conformed to the classical descriptions of *T. thiooxidans* and *T. thioparus* were isolated locally. The isolation and properties of a newly described facultative autotroph, *T. intermedius*, have been described elsewhere (28, 30). Stock cultures of thiobacilli were maintained in shaken flasks of mineral thiosulfate medium, supplemented with 0.05% (w/v) yeast extract for *T. intermedius*. The cultures, incubated at 30 C, were periodically neutralized with sterile Na_2CO_3 until thiosulfate had been exhausted, and were then stored at 10 C.

H. eutropha strain 335 was kindly provided by Diane Davis. Stock cultures were maintained on yeast extract-agar slants.

Media and conditions of cultivation. The blue-green algae were grown on the mineral medium of Hughes, Gorham, and Zehnder (18), modified by increasing the concentration of $NaNO_3$ threefold. Maximal growth rates and cell yields in liquid cultures required aeration with a mixture of 95% air-5% CO_2, and, in order to maintain the *p*H in a favorable range, the concentration of the Na_2CO_3 in the medium was increased 20-fold for *A. nidulans* and *C. peniocystis*, and 125-fold for *G. alpicola*. To prevent the formation of an insoluble precipitate, sodium carbonate was separately sterilized and added to the medium after autoclaving.

Inocula of blue-green algae for incorporation experiments were grown in 125-ml Erlenmeyer flasks containing 25 ml of mineral medium. These cultures were gassed with a mixture of 95% air-5% CO_2 and agitated in a Bronwell photosynthetic Warburg bath, maintained at 32 C and illuminated from below with seven 40-w tungsten lamps. The light intensity at the surface of the vessels was 500 ft-c. Larger quantities of organisms were grown in 1-liter Roux bottles fitted with the gassing head and sampling device described by Cohen-Bazire et al. (11). Cultures were gassed with a mixture of 95% air-5% CO_2 and incubated in a narrow rectangular Perspex water bath which was maintained at 32 C. The bath was illuminated bilaterally with cool white fluorescent strip lamps; the light intensity at both faces of the bottle was 750 ft-c. Growth was measured turbidimetrically on samples removed from cultures. A Klett-Summerson colorimeter fitted with a Kodak Wratten gelatin filter number 88A was used. The relationship between dry weight and turbidity was linear over the range from 25 to 200 Klett units.

The thiobacilli were grown in Baalsruds' medium (7) supplemented with 0.002% $FeCl_3$ and 2 ml of Pfennig's trace metals mixture (35) per 100 ml of medium. The initial *p*H of the medium was adjusted to 6.8 for *T. intermedius* and *T. thioparus* and 5.8 for *T. thiooxidans*. Growth experiments were carried out in 500-ml Erlenmeyer flasks containing 100 ml

of medium. A mixture of 95% air-5% CO_2 was passed through the cultures, which were incubated in a 30 C reciprocating shaker bath. For preparation of cell-free extracts, organisms were grown in 3-liter Fernbach flasks incubated at 30 C on a rotary shaker.

H. eutropha strain 335 was grown in the medium of Doudoroff (13). Autotrophic cultures were grown on solidified medium (2% Difco agar, w/v) in petri plates under an atmosphere of 80% H_2, 10% CO_2, and 10% O_2 at 30 C. Heterotrophic cultures of this organism were grown in 500-ml Erlenmeyer flasks containing 100 ml of Doudoroff's medium supplemented with 0.25% (w/v) Na lactate and were incubated at 30 C on a rotary shaker.

The growth of the chemoautotrophs was measured turbidimetrically at 660 mμ with a Klett-Summerson colorimeter. The relationship between dry weight and turbidity was linear over the range 10 to 100 Klett units.

Incorporation of ^{14}C-labeled organic compounds during growth. Cultures of blue-green algae grown to a density of 0.08 mg (dry weight)/ml were transferred aseptically to rectangular glass culture vessels (2 × 4.5 × 15 cm) fitted with a sampling and gassing device, and incubated as described previously for cultures in Roux bottles. ^{14}C-labeled organic compounds were added as freshly prepared heat- or filter-sterilized solutions containing 5 μc of ^{14}C; the final concentration of the organic substrate was 5 mM unless otherwise stated.

Cultures of *T. thioparus* and *T. thiooxidans* were grown to a density of 0.20 mg (dry weight)/ml in 100 ml of thiosulfate-mineral salts medium. Samples of 10 ml of neutralized culture were used to inoculate 90 ml of fresh medium. Labeled organic compounds were added as freshly prepared heat- or filter-sterilized solutions containing 5 μc of ^{14}C. The final concentrations of organic substrates in cultures of *T. thioparus* and *T. thiooxidans* ranged from 0.2 to 2 mM.

To avoid long lag periods, cultures of *T. intermedius* were preadapted by growing them in the presence of the organic substrates to be tested. Growth experiments were performed as described for the obligately autotrophic thiobacilli. ^{14}C-labeled organic compounds containing 5 μc of ^{14}C were added to cultures of the facultative autotroph as freshly prepared heat- or filter-sterilized solutions to give a final concentration of 5 mM.

The incorporation of isotope was always followed for at least two generations. Culture samples were removed aseptically and drawn through cellulose plastic filters (Millipore Corp., Bedford, Mass.) with an average pore size of 0.22 μ. The cells, retained on the filter, were washed twice with a solution of the organic compound in the presence of which they had been grown. Each wash consisted of 5 ml of a solution of the nonradioactive compound, at a concentration 10 times its initial concentration in the culture medium. The cells were then washed twice with distilled water. The membrane filters were dried at room temperature and glued onto aluminum planchets. At the beginning of each experiment, duplicate samples of the whole culture were plated on aluminum planchets to determine total radioactivity.

Samples were counted in a Nuclear-Chicago gas-flow end-window planchet counter, model D-47. Self-absorption was negligible, and the counts were corrected for background radiation. The efficiency of counting was approximately 25%.

At the conclusion of an experiment, undiluted samples of each culture were streaked on 0.5% yeast extract-agar to detect heterotrophic contamination. This was rare; when it occurred, the results of the experiment were discarded.

Preparation and resolution of cellular amino acids. Organisms from between 50 and 100 ml of cultures grown in the presence of a ^{14}C-labeled organic compound were harvested after at least a fourfold increase in density by centrifuging at 14,000 × *g* for 15 min. The cells were washed twice with a solution of the organic compound in the presence of which they had been grown; each wash consisted of 10 to 20 ml of a solution of the nonradioactive organic compound, 10 times more concentrated than that used in the culture medium. The cells were then washed twice with distilled water. The cell pellets were resuspended in 2 ml of distilled water, divided into two equal parts, and transferred to glass vials containing 1 ml of 12 N HCl. The vials were sealed under vacuum and heated in an autoclave overnight at 15 lb/in².

The hydrolysates were dried under vacuum, and the residues were taken up in 1 ml of warm water (70 to 80 C) and filtered through Whatman no. 1 paper. The filtrates were extracted twice with an equal volume of ethyl ether, and the ether extracts were discarded. The aqueous layer was dried under vacuum, and the residue was taken up in 1 ml of 20% (v/v) isopropanol. Samples containing isotope in excess of 10,000 counts/min were applied to large sheets of Whatman no. 1 paper. The chromatograms were developed with *n*-butanol-water-acetic acid (500:500:120, v/v) and phenol-water (400:100, v/v) as solvent systems.

A control chromatogram was prepared by using an isotope-free hydrolysate, developed under identical conditions, sprayed with 0.25% (w/v) ninhydrin in acetone and heated at 60 C for 15 min. The position of the amino acids on ^{14}C-labeled chromatograms was established by comparing the ^{14}C-labeled chromatograms with the control chromatogram, after spraying the former very lightly with 0.02% ninhydrin (w/v) in acetone. The ninhydrin-positive areas were cut out and the amino acids were eluted with 75% acetone in water. The eluates were transferred to glass scintillation vials and were dried under vacuum. The residues were taken up in 1-ml volumes of water, mixed with 9 ml of Bray's solution, and counted in a Nuclear-Chicago Mk I scintillation counter with an efficiency of 60 to 70%. By using ^{14}C-labeled aspartate, glutamate, and an amino acid mixture of known composition and constant carbon specific activity, it was established that the overall efficiency of elution was approximately 80%. The individual amino acids were eluted with comparable efficiency,

and aspartate and glutamate were completely resolved by these techniques.

Preparation of cell-free extracts. Organisms were harvested during logarithmic growth at a density of 0.3 mg (dry weight)/ml for blue-green algae and 0.15 mg (dry weight)/ml for the chemoautotrophs. Petri plates of autotrophically grown *H. eutropha* were harvested 36 hr after inoculation, at which time a thin film of organisms covered the agar surface. The organisms were washed twice in 20 ml of 0.05 M phosphate buffer (the *p*H of the buffer was 7.5 for the blue-green algae, 6.9 for *T. thioparus*, *T. intermedius*, and *H. eutropha*, and 6.2 for *T. thiooxidans*). The washed organisms were resuspended in the same buffer to a density of approximately 10 mg (dry weight)/ml. Crude extracts were prepared by ultrasonic disruption under an atmosphere of N_2 with a 10-kc Raytheon ultrasonic generator operating at full power for 6 to 8 min at 8 C. Whole cells and debris were removed by centrifuging at 8,000 × *g* for 10 min. The crude supernatant fluid was then centrifuged at 38,000 × *g* for 30 min. The supernatant fluid was decanted, and the pellet was carefully resuspended in 5 ml of 0.05 M buffer at the appropriate *p*H. In every instance, extracts were prepared from two different cultures of the organisms examined.

Enzyme assays. Isocitric acid dehydrogenase activity was measured by the method of Kornberg (21), malic acid dehydrogenase by the technique of Ochoa (34), α-ketoglutaric acid dehydrogenase by the technique of Amarasingham and Davis (5), and succinic acid dehydrogenase by the beef cytochrome *c* method of Massey (32). Reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase activity was measured in a reaction mixture containing: 200 μmoles of phosphate buffer, *p*H 7.0; 0.2 μmole of $NADH_2$; crude extract, 0.6 to 1.0 mg of protein, and water to a final volume of 3 ml. Reaction rates were determined at the appropriate wavelengths in a Gilford multichannel recording spectrophotometer, with a 1-cm light path.

Chemicals and protein estimation. Uniformly labeled ^{14}C-glucose, ^{14}C-fructose, ^{14}C-glutamate, ^{14}C-aspartate, and pyruvate-*3*-^{14}C, acetate-*1,2*-^{14}C, malate-*3*-^{14}C, succinate-*2,3*-^{14}C, and leucine-*1*-^{14}C, were obtained from New England Nuclear Corp., Boston, Mass. or Calbiochem, Los Angeles, Calif. Protein was determined by the Lowry method (31), with crystalline bovine serum albumin used as standard.

RESULTS AND DISCUSSION

Design of incorporation experiments. The first goal of this work was to examine the range of organic compounds assimilable by obligate autotrophs, and the extent to which they are used for the synthesis of cell material. Meaningful comparative data on incorporation require measurements of *the differential rate of uptake of each compound during the course of exponential growth.* It follows that exponential growth conditions must be maintained throughout the course of each experiment. Furthermore, the organic compounds should be furnished at relatively high concentrations (provided that such concentrations are not growth-inhibitory) in order to ensure that uptake continues over the entire period of the experiment. The use of a high substrate concentration is also desirable for another reason: uptake of some organic compounds may be strongly concentration-dependent. These technical points have been ignored in earlier studies on the assimilation of organic compounds by obligate autotrophs. In some cases (e.g., 9, 10, 19), the compounds have been supplied in trace amounts, in others very dense (and essentially resting) cell suspensions have been used (e.g. 17; G. G. Still, Ph.D. Thesis, Oregon State Univ., Corvallis, 1965). The existence of uptake can be ascertained by such experiments, but the data cannot be evaluated and compared in quantitative terms.

Incorporation of organic compounds during photosynthetic growth by blue-green algae. The uptake of organic compounds by blue-green algae growing exponentially in the light proceeded at a constant differential rate which was characteristic for each compound. Typical results obtained with *A. nidulans* and *G. alpicola* are shown in Fig. 1 and 2. None of the organic compounds examined measurably increased the growth rate when added to a culture growing

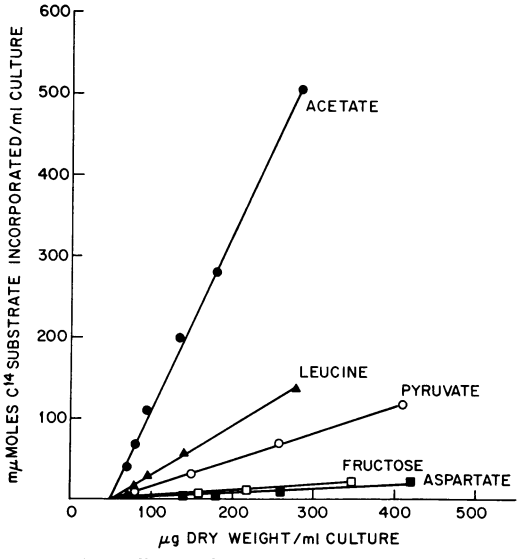

FIG. 1. *Differential rates of incorporation of five organic compounds by Anacystis nidulans during exponential photosynthetic growth. Curves are a combination of the results of several experiments.*

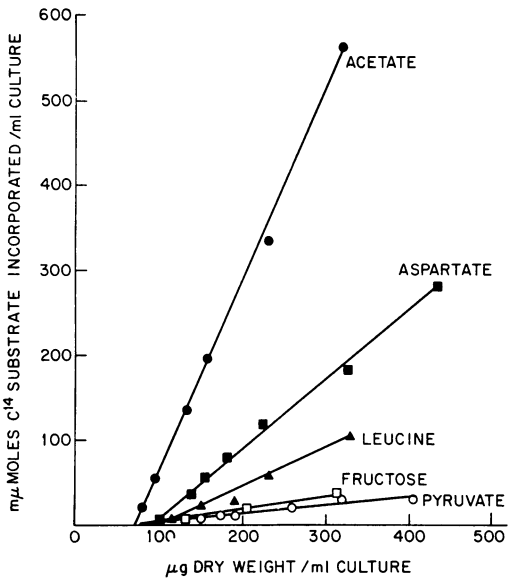

FIG. 2. *Differential rates of incorporation of five organic compounds by Gloeocapsa alpicola during exponential photosynthetic growth. Curves are a combination of the results of several experiments.*

in a mineral medium. In the dark, uptake of organic compounds was negligible.

Data on the incorporation of nine different organic compounds by the three strains of blue-green algae are presented in Table 2. In Table 2 (and 4), measured differential rates of incorporation (millimicromoles of organic substrate assimilated per milligram of dry weight increase) have been used to calculate the fractional contribution of carbon from each organic substrate to newly synthesized cell carbon, assuming that carbon accounts for 50% of the cellular dry weight. This value, expressed as milligrams of organic carbon incorporated per 100 mg of newly synthesized cell carbon, permits direct comparison of the effectiveness with which each compound tested contributes to biosynthesis.

The patterns of organic carbon assimilation by the three blue-green algae are remarkably similar. In every case, acetate was most effectively assimilated, contributing approximately 10% to newly synthesized cell carbon; assimilation of sugars, pyruvate, and dicarboxylic acids was considerably smaller. Of the three amino acids examined (glutamate, aspartate, and leucine), leucine was effectively incorporated, whereas little glutamate was assimilated. Aspartate was effectively assimilated by *G. alpicola*, but poorly assimilated by the other two strains. The magnitude of leucine assimilation (and of aspartate assimilation by *G. alpicola*) suggests that endogenous synthesis may be almost completely suppressed when an exogenous supply of the amino acid in question is available.

Table 3 shows how initial concentration affected the rate of incorporation of several organic compounds by *A. nidulans* and *G. alpicola*. The rate of acetate uptake was essentially independent of concentration, whereas the rate of glucose uptake was directly proportional to concentration. The results obtained for acetate are of particular interest. Regardless of concentration, this organic compound cannot contribute more than 10% of the newly synthesized carbon of the cell, although it is more rapidly assimilated than any other organic compound tested. Hence, even

TABLE 2. *Magnitude of incorporation of organic compounds by blue-green algae during photosynthetic growth*

Organic substrate (initial concn: 5 mM)	Amt (mg) of organic carbon incorporated per 100 mg of newly synthesized cell carbon[a]		
	Anacystis nidulans	*Coccochloris peniocystis*	*Gloeocapsa alpicola*
Glucose	0.6	ND[b]	ND
Fructose	1.2	1.7	2.0
Pyruvate	3.0	5.6	0.6
Acetate	10.4	8.2	10.8
Succinate	0.4	1.3	1.0
Malate	0.2	1.4	1.0
Glutamate	1.0	0.3	1.8
Aspartate	0.5	0.2	9.9
Leucine	8.0	6.7	6.7

[a] Calculated from differential rates of incorporation, assuming that carbon accounts for 50% of the cellular dry weight.
[b] Not determined.

TABLE 3. *Effect of concentration on the differential rates of incorporation of organic compounds by blue-green algae during photosynthetic growth*

Substrate concn (mM)	Amt (mμmoles) of organic substrate assimilated per mg of dry wt increase				
	Anacystis nidulans			*Gloeocapsa alpicola*	
	Glucose	Acetate	Leucine	Aspartate	Acetate
0.1	ND[a]	ND	103	ND	ND
1.0	ND	2,160	206	ND	ND
5	42	2,150	555	965	2,250
25	195	2,620	ND	4,650	2,280
50	588	ND	ND	ND	ND

[a] Not determined.

in the presence of a readily assimilable organic compound, the blue-green algae remain overwhelmingly dependent on the assimilation of CO_2 to meet gross carbon requirements.

Incorporation of organic compounds during chemoautotrophic growth of thiobacilli. Figure 3 shows the differential rates of incorporation of several organic compounds by *T. thiooxidans* during chemoautotrophic growth at the expense of thiosulfate. With the thiobacilli, acid production resulting from thiosulfate oxidation caused a fall of *p*H in the medium during growth, and this in turn affected the differential rates of incorporation of organic compounds. For this reason, differential rates were calculated from the initial slopes of the curves relating incorporation to growth.

Table 4 summarizes data on the incorporation of nine different organic compounds into cell material by *T. thiooxidans* and *T. thioparus* (obligate autotrophs) and by *T. intermedius* (facultative autotroph). As the two obligately autotrophic bacteria proved to be somewhat more sensitive to organic compounds than the blue-green algae, it was necessary to provide lower initial concentrations (in most cases 2 mM). Despite this difference in experimental design, the relative magnitudes of incorporation were in general similar to those observed with the blue-green algae. Acetate was used more effectively than fructose or pyruvate, and again accounted for about 10% of newly synthesized cell carbon. Succinate was poorly incorporated by *T. thiooxidans*, but *T. thioparus* incorporated it slightly more effectively than acetate. Amino acids (glutamate, aspartate, and leucine) were incorporated effectively by both species; assimilation of glutamate was considerably greater than that observed with the blue-green algae. The effect of the initial concentration of acetate on the differential rate of its incorporation was examined only for *T. thiooxidans*, the differential rate was unchanged over a concentration range of 0.2 to 1.0 mM. It therefore seems probable that the obligately autotrophic thiobacilli, like the blue-green algae, can use acetate to supply no more than 10% of their total carbon requirements. None of the organic compounds caused a measurable increase in the rate of growth of *T. thiooxidans* and *T. thioparus* over the rate of growth observed in a mineral medium.

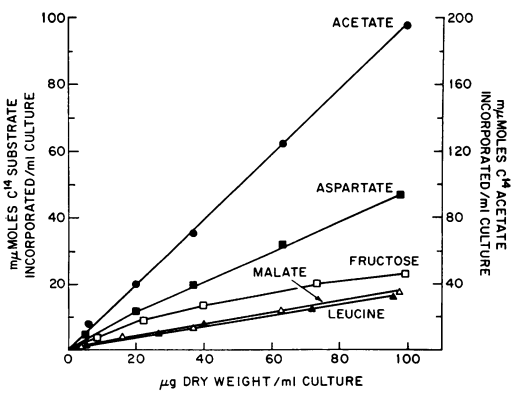

FIG. 3. *Differential rates of incorporation of five organic compounds by Thiobacillus thiooxidans during exponential chemoautotrophic growth.*

TABLE 4. *Magnitude of incorporation of organic compounds by thiobacilli during chemoautotrophic growth*

Organic substrate	Amt (mg) of organic carbon incorporated per 100 mg of newly synthesized cell carbon[a]		
	Thiobacillus thiooxidans	*T. thioparus*	*T. intermedius*
Glucose	ND[b]	ND	39.4 (5)
Fructose	2.9 (2)	ND	ND
Pyruvate	4.0 (1)	2.6 (2)	4.6 (5)
Acetate	8.8 (0.2)	9.2 (2)	43.6 (5)
Succinate	0.8 (0.5)	11.6 (2)	86.0 (5)
Malate	3.7 (2)	ND	ND
Glutamate	3.0 (2)	7.2 (2)	87.8 (5)
Aspartate	4.3 (2)	7.3 (2)	ND
Leucine	2.6 (2)	2.9 (2)	ND

[a] Calculated from differential rates of incorporation, assuming that carbon accounts for 50% of the cellular dry weight. Figures in parenthesis give the initial concentration of the organic substrate in the growth medium (mM).

[b] Not determined.

Incorporation experiments with *T. intermedius* (also shown in Table 4) yielded entirely different results. Glucose, acetate, succinate, and glutamate were massively incorporated into cell material, accounting for 40 to 88% of newly synthesized cell carbon. Although the growth of *T. intermedius* in a mineral medium supplemented with a single assimilable organic compound is strictly dependent on the presence of the inorganic oxidizable substrate, thiosulfate, the growth rate is substantially higher in the presence of glucose, acetate, succinate, or glutamate than in a mineral medium.

Intercellular distribution of assimilated organic carbon. Gross analyses of the intracellular distribution of organic carbon assimilated by the three blue-green algae were performed by the methods of Roberts et al. (37). The isotopic distribution in cells grown with ^{14}C-acetate and ^{14}C-pyruvate was similar to that previously reported by Hoare and Moore (17) for blue-green algae grown with ^{14}C-

acetate: most of the radioactivity was recovered in the lipid and protein fractions. However, radioactivity from glutamate was recovered almost entirely in the protein fraction. Radioactivity from hexose, malate, succinate, and aspartate was more or less uniformly distributed among all major cell fractions.

To examine more precisely the fate of assimilated carbon, the distribution of radioactivity derived from the organic substrates in the amino acid residues of cell proteins was determined. Cells which had incorporated radioactivity from an organic substrate were subjected to acid hydrolysis, and the amino acids present in such hydrolysates were partially separated by two-dimensional paper chromatography. Areas containing amino acids were detected with ninhydrin, and the amount of isotope in each ninhydrin-positive area was determined. The method of chromatography employed was generally satisfactory for amino acid separation, but yielded two unresolved clusters: leucine-isoleucine-methionine and phenylalanine, and histidine-lysine-arginine. In interpreting the occurrence of radioactivity in these unresolved clusters, we have assumed that the pathways of amino acid biosynthesis in blue-green algae and thiobacilli are basically similar to those of heterotrophic bacteria. If this assumption is correct, it follows that methionine will not be radioactive when aspartate and threonine are not, since all three amino acids are derived from a common precursor (oxaloacetate). Similarly, lysine and isoleucine should not be radioactive when aspartate, threonine, and alanine are not. The probable identity of the radioactive amino acid(s) in confluent spots could be determined with reasonable certainty (granting the basic premise), since in every case at least two biosynthetically related amino acids were clearly resolved.

The incorporation of isotope from ^{14}C-acetate into amino acids by the obligate autotrophs was essentially restricted to the glutamic acid family and leucine (Table 5). Aspartate, threonine, and the other clearly resolved amino acids each accounted for less than 1% of the total isotope recovered from the chromatograms. It is evident that these organisms use acetate carbon for the synthesis of a surprisingly limited number of amino acids. Our data confirm and extend the results obtained by others for blue-green algae (17) and for an obligately autotrophic *Thiobacillus* (G. G. Still, Ph.D. Thesis, Oregon State Univ., Corvallis, 1965).

The incorporation pattern of acetate carbon suggested that the obligate autotrophs lack one or more of the enzymes required for the conversion of acetate to oxaloacetate via α-ketoglutarate (Fig. 4). If this assumption is correct, the isotope derived from the assimilation of ^{14}C-glutamate should be restricted to the amino acids of the glutamic acid family. The data of Table 6 confirm this inference. Leucine, aspartate, and threonine do not receive significant radioactivity from ^{14}C-glutamate.

The labeling patterns resulting from the incorporation of ^{14}C-pyruvate (Table 7) are also distinctive. Radioactivity was essentially restricted to valine, alanine, leucine, and the amino acids of the glutamic acid family. Aspartate, threonine, serine, and glycine did not incorporate radioactivity from pyruvate. The failure of radioactivity to enter aspartate and threonine shows that

TABLE 5. *Distribution[a] of radioactive carbon among the cellular amino acids after autotrophic growth in the presence of ^{14}C-acetate*

Amino acid	Organism					
	Anacystis nidulans	*Coccochloris peniocystis*	*Gloeocapsa alpicola*	*Thiobacillus thiooxidans*	*T. thioparus*	*T. intermedius*
Leucine, isoleucine, phenylalanine, methionine	34	26	33	33	29	43
Valine						6
Proline	14	13	14	21	14	5
Tyrosine						1
Alanine						6
Threonine						6
Glutamate	43	51	44	36	42	11
Aspartate						8
Cystine						1
Serine, glycine						6
Lysine, arginine, histidine	9	10	9	10	15	7

[a] Expressed as percentage of radioactivity recovered from chromatograms.

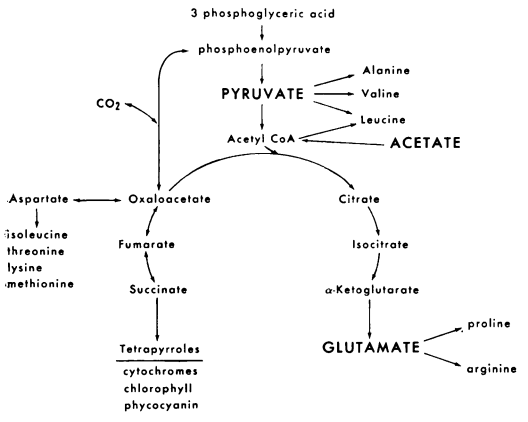

FIG. 4. *Schematic representation of certain intermediary metabolic pathways in the obligate autotrophic blue-green algae and thiobacilli, to illustrate the flow of carbon from exogenous acetate, pyruvate, and glutamate into amino acids.*

TABLE 6. *Distribution[a] of radioactive carbon among the cellular amino acids after autotrophic growth in the presence of ^{14}C-glutamate*

Amino acid	Organism		
	Anacystis nidulans	*Thiobacillus thiooxidans*	*T. thioparus*
Proline	17	26	21
Glutamate	73	58	57
Lysine, arginine, histidine	10	16	22

[a] Expressed as percentage of radioactivity recovered from chromatograms. No radioactivity was detected in the following amino acids: leucine, isoleucine, phenylalanine, methionine, valine, tyrosine, alanine, threonine, aspartate, cystine, serine, and glycine.

both blue-green algae and thiobacilli are unable to use pyruvate as a precursor of oxaloacetate; presumably, phosphoenolpyruvate is the immediate precursor of oxaloacetate. In fact, enzymological data have shown that *T. thiooxidans* contains phosphoenolpyruvate carboxylase, but lacks pyruvate carboxylase and phosphoenolpyruvate synthetase (43). The labeling patterns from pyruvate observed in *T. thioparus* and the blue-green algae suggests that they have the same constitution with respect to the enzymes of pyruvate metabolism.

The patterns of incorporation of radioactivity into amino acids from pyruvate and acetate by *T. intermedius* (Tables 5 and 7) are markedly different from the very uniform pattern characteristic of the obligate autotrophs. In this facultative autotroph, carbon from radioactive acetate and pyruvate was distributed among all cellular amino acids.

The experiments described above lead to one major biochemical conclusion: none of the obligate autotrophs examined has an operative tricarboxylic acid cycle. In every case, the reactions of the cycle appear to be interrupted at the level of α-ketoglutarate oxidation. The one facultative autotroph examined, *T. intermedius*, does not share this metabolic defect.

Enzymatic activities of cell-free extracts. To confirm and extend the conclusions derived from incorporation patterns, cell-free extracts were prepared from the five obligate autotrophs and from two facultative autotrophs, and were examined for the presence of four enzymes operative in the tricarboxylic acid cycle and of $NADH_2$ oxidase (Table 8). The facultatively autotrophic hydrogen bacterium, *H. eutropha*, was grown both autotrophically and heterotrophically, and enzymatic activities were determined in extracts prepared from both types of cells.

Isocitric dehydrogenase, selected as an enzyme representative of the initial reactions of the tricarboxylic acid cycle, was present at high levels in all the organisms examined. Its level in the obligately autotrophic thiobacilli is not significantly different from that in *T. intermedius* and

TABLE 7. *Distribution[a] of radioactive carbon among the cellular amino acids after autotrophic growth in the presence of ^{14}C-pyruvate*

Amino acid	Organism			
	Anacystis nidulans	*Coccochloris peniocystis*	*Thiobacillus thiooxidans*	*T. intermedius*
Leucine, isoleucine, phenylalanine, methionine	42	36	39	19
Valine	14	3	20	15
Proline	6	7	7	2
Tyrosine				6
Alanine	14	14	15	18
Threonine				5
Glutamate	18	23	12	18
Aspartate				7
Cystine				2
Serine, glycine				5
Lysine, arginine, histidine	6	7	7	6

[a] Expressed as percentage of radioactivity recovered from chromatograms.

TABLE 8. *Activities of enzymes in cell-free extracts of obligate and facultative autotrophs*

Enzyme	Prepn examined[a]	Specific activity (mμmoles of substrate oxidized per min per mg of protein)							
		Anacystis nidulans	*Coccochloris peniocystis*	*Gloeocapsa alpicola*	*Thiobacillus thiooxidans*	*T. thioparus*	*T. intermedius* A[b]	*Hydrogenomonas eutropha*	
								A[b]	H[b]
Isocitric dehydrogenase	S	36	36	36	80	74	103	101	73
α-Ketoglutaric dehydrogenase	S + P	0	0	0	0	0	0.8	1.0	1.5
Succinic dehydrogenase	S	6.9	2.1	3.4	7.9	7.5	70	90	21
Malic dehydrogenase	S	7.8	7.1	17	11.2	28	1.6×10^3	8.3×10^3	21×10^3
$NADH_2$ oxidase	Crude extract	0	0	0	0	0	12	22	15

[a] The crude extracts were resolved into supernatant (S) and particulate (P) fractions as described in Materials and Methods.

[b] A = autotrophically grown; H = heterotrophically grown.

H. eutrophia, whereas its level in the blue-green algae is about 50% lower.

α-Ketoglutaric dehydrogenase was readily demonstrated in extracts of *T. intermedius* and *H. eutropha*, although its specific activity was very low relative to those of the other tricarboxylic acid cycle enzymes examined. However, all attempts to demonstrate this enzyme in extracts of the obligate autotrophs failed. Control experiments with mixed extracts showed that the negative results could not be attributed to the presence of an inhibitor of α-ketoglutaric dehydrogenase activity in the extracts of the obligate autotrophs.

Succinic and malic dehydrogenases were readily detectable in extracts of all seven organisms. However, there was a clearcut difference between the obligate and the facultative autotrophs with respect to the specific activities of these enzymes. The difference was particularly striking for malic dehydrogenase; the specific activity of this enzyme was approximately two orders of magnitude lower in the obligate autotrophs than in the facultative autotrophs. These facts are readily intelligible in metabolic terms. Since the obligate autotrophs do not possess α-ketoglutaric dehydrogenase, the reaction-sequence from succinate to oxaloacetate must operate in reverse in these organisms, as shown in Fig. 4, thus fulfilling a purely biosynthetic role (5). Under these circumstances, succinic and malic dehydrogenase can adequately serve their metabolic function at relatively low levels of specific activity (14, 15).

$NADH_2$ oxidase activity was readily demonstrable in crude extracts of *T. intermedius* and *H. eutropha*, but all attempts to demonstrate it in extracts of the obligate autotrophs failed. It therefore follows that the blue-green algae, *T. thiooxidans*, and *T. thioparus* cannot mediate a transfer of electrons from $NADH_2$ to oxygen. The absence of $NADH_2$ oxidase activity makes it possible to explain in biochemical terms earlier observations on the respiratory behavior of intact cells of these organisms. The endogenous respiration of blue-green algae is very low, and is only marginally increased by the provision of exogenous organic substrates (23). The obligately autotrophic thiobacilli do not consume a significant quantity of oxygen in the presence of organic substrates, although under the same conditions they rapidly oxidize reduced sulfur compounds to completion. *T. intermedius*, on the other hand, consumes oxygen at a rate significantly higher than the endogenous rate when furnished with organic substrates (30).

Biochemical basis of obligate autotrophy. The enzymatic analyses revealed consistent differences between obligate and facultative autotrophs with respect to three central metabolic functions. The obligate autotrophs are characterized by: (i) absence of α-ketoglutaric dehydrogenase; (ii) absence of $NADH_2$ oxidase; and (iii) unusually low levels of malic and succinic dehydrogenase. We shall now consider whether these metabolic abnormalities are necessary and sufficient to explain the phenomenon of obligate autotrophy.

In order to support heterotrophic growth, an organic compound must fulfill two different metabolic functions. It must serve as a general

precursor of the carbon compounds of the cell, and it must be dissimilable in a manner that permits the generation of the adenosine triphosphate (ATP) and reducing power required to drive the biosynthetic reactions of the cell. Our observations on the metabolic potentialities of blue-green algae and thiobacilli suggest that certain organic compounds should be able to fulfill the first of these two functions. Either a metabolic precursor of phosphoenolpyruvate, or a C4 dicarboxylic acid in conjunction with acetate, should substitute adequately with respect to cellular carbon requirements for 3-phosphoglyceric acid, the major product of autotrophic CO_2 fixation.

The basic obstacle to heterotrophic growth in the obligate autotrophs appears to be their inability to couple the breakdown of organic substrates with the generation of ATP, as a result of the absence of $NADH_2$ oxidase. This factor is far more important than the blockage of the tricarboxylic acid cycle at the level of α-ketoglutarate oxidation. An incomplete oxidation of organic substrates through the initial reactions of the cycle (with excretion of α-ketoglutarate) could provide a supply of reduced pyridine nucleotide; it is the inability to use this reduced pyridine nucleotide for ATP generation which prevents fulfillment of the energy requirements for biosynthesis. In fact, the only conceivable way in which the obligate autotrophs could generate ATP during the metabolism of organic compounds would be by substrate-level phosphorylations accompanying glycolysis, a process that might support growth if they could mediate uncoupled transfer of electrons to oxygen (for example, via flavoprotein enzymes).

Physiological and evolutionary implications of the absence of $NADH_2$ oxidase in obligate autotrophs. The absence of $NADH_2$ oxidase in blue-green algae raises no problems for the interpretation of their autotrophic mode of life. In any oxygen-evolving phototroph, the necessary balance between the generation of reduced pyridine nucleotide and of ATP can be achieved by appropriate regulation of the relative rates of cyclic and noncyclic photophosphorylation (6).

However, the absence of $NADH_2$ oxidase from *T. thiooxidans* and *T. thioparus* has an important bearing on the interpretation of their autotrophic mode of life. It implies that NAD is not a component in the chain of electron transport at any step in the oxidation of reduced inorganic sulfur compounds to sulfate. In this context, it is of some interest to note that all attempts to demonstrate a direct reduction of NAD coupled with the oxidation of sulfide, thiosulfate, tetrathionate, and trithionate by cell-free extracts of thiobacilli have failed (J. London, *unpublished data*), whereas a coupling of these oxidations with the reduction of a *c*-type cytochrome is readily observed (29). For purely thermodynamic reasons, a direct reduction of NAD by electrons derived from the inorganic energy source is impossible in chemoautotrophs oxidizing NO_2^-, NH_4^+, or Fe^{++}. We therefore suggest that the generation of reduced pyridine nucleotide by the ATP-mediated reversal of electron transport, which has been experimentally demonstrated in *Nitrobacter* (2) and *Thiobacillus novellus* (3), may be a general and distinctive property of all chemoautotrophs except the hydrogen bacteria.

The hydrogen-oxidizing chemoautotrophs clearly represent a special case, since the low potential of the hydrogen-hydrogen ion couple necessitates the presence, in the transport chain to oxygen, of a carrier at the potential level of pyridine nucleotides. $NADH_2$ oxidase may thus be an essential enzyme for the autotrophic metabolism of hydrogen bacteria. This could explain why the hydrogen bacteria are the only group of chemoautotrophs in which autotrophy is invariably facultative. In chemoautotrophs which transfer electrons from inorganic substrates to the carrier chain at the cytochrome level and generate $NADH_2$ by reverse electron transport, the elimination of $NADH_2$ oxidase would have an obvious selective advantage, since this enzyme constitutes a potential threat to the conservation of reducing power. However, if such an evolutionary loss of function occurred, the chemoautotroph would destroy the bridge that enables it to use organic compounds as sources of energy, and it would be permanantly dependent on its oxidizable inorganic substrate for the fulfilment of its energetic needs. The loss of $NADH_2$ oxidase can therefore be regarded as an essential step in the evolution of a fully specialized chemoautotroph. This loss would immediately cause a profound change in the metabolic role of the enzymes of the tricarboxylic acid cycle. These enzymes would no longer serve a function in energy-yielding metabolism, but would still be required for biosynthetic purposes. A cyclic flow is, however, unnecessary to meet biosynthetic needs, provided that succinate (required for porphyrin synthesis) can be generated from oxaloacetate (Fig. 4). Under such circumstances, α-ketoglutaric dehydrogenase is an unnecessary enzyme of the cycle. The metabolic peculiarities of obligate chemoautotrophs can thus be logically explained in evolutionary terms: they have arisen from heterotrophic ancestors by successive elimination of two

enzymes which play key roles in heterotrophic metabolism.

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