Arch. Microbiol. 100, 163—178 (1974)

Growth and Metabolism of the Obligate Photolithotroph *Chlorobium thiosulfatophilum* in the Presence of Added Organic Nutrients*

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Received July 22, 1974

Abstract. Growth of *Chlorobium vibrioforme* f. *thiosulfatophilum* NCIB 8327 could be monitored by measurement of turbidity (E_{600}); absorbance at 745 and 665 nm; increase in methanol-extractable pigment (E_{660}); fixation of $^{14}CO_2$; and titration of thiosulphate and sulphide in the medium. Growth could be inhibited by formate, methionine, tryptophan, tyrosine, threonine, serine and glycine, but not by 14 other amino acids, shikimic acid, some alcohols, sugars or acetate. Inhibition could sometimes be relieved by the presence of other amino acids. This was probably partly due to restoration of normal internal amino acid requirements by "feeding", and partly because uptake of amino acids appeared to show some competition for two or more low specificity uptake systems. Numerous ^{14}C-labelled amino acids, formate and glucose were shown to be photoassimilated by *Chlorobium*, and the labelling patterns obtained provided information on its pathways of intermediary biosynthesis. Growth inhibition by threonine could be related to the probable presence of a normal branched pathway for the synthesis of the aspartate family of amino acids, with an aspartokinase enzyme subject to strong inhibition by threonine and lysine, separately and in combination.

Key words: Chlorobium thiosulfatophilum — Amino Acid Metabolism — Growth Inhibition — Photoheterotrophy.

Chlorobium has been known since 1906 (Nadson, 1906, 1912) and details of the general stoichiometry of sulphur compound oxidation and carbon dioxide assimilation known for many years (van Niel, 1931; Larsen, 1953). *Chlorobium* species are characterized as being obligately anaerobic and absolutely dependent on light, carbon dioxide and oxidizable sulphur or hydrogen for obligately photosynthetic growth. Although the photoassimilation of acetate and carboxylation of propionate by *Chlorobium* have been reported (Larsen, 1953; Nesterov *et al.*, 1965; Pfennig, 1967) little is known about intermediary carbon metabolism following the initial fixation of carbon dioxide by the Calvin cycle or reductive tricarboxylic acid cycle (Kelly, 1971).

* Dedicated to Professor C. B. van Niel and the "Archiv für Mikrobiologie", which published his historic studies on purple and green bacteria in 1931.

This paper describes experiments which provide information on the response of *Chlorobium thiosulfatophilum* to organic nutrients in its environment.

Materials and Methods

Organism and Culture Conditions. Chlorobium thiosulfatophilum NCIB 8327 was obtained from Prof. J. Lascelles in 1967. This strain should now be known as *Chlorobium vibrioforme* forma specialis *thiosulfatophilum* (Larsen) Pfennig and Trüper 1971 (Pfennig and Trüper, 1971; DSM, 1973). It was maintained with continuous illumination at 25°C in flat bottles completely filled with a medium containing (g/l distilled water): KH_2PO_4, 1.0; NH_4Cl, 1.0; $MgCl_2 \cdot 6H_2O$, 0.5; $Na_2S_2O_3 \cdot 5H_2O$, 1.0; $Na_2S \cdot 9H_2O$, 0.12; $NaHCO_3$, 2.0; trace metals solution (Pfennig and Lippert, 1966), 20 ml; 0.1 N HCl, 7.5 ml. For growth experiments, many replicate 100 ml bottles with a 5 or 10% (v/v) inoculum were set up and successive bottles sacrificed for analysis throughout the growth period. For ^{14}C-incorporation experiments, a 500 ml culture in early growth was rapidly distributed among a series of 20 ml tubes or 40 ml bottles already containing the labelled compounds. Some bulk cultures on $^{14}CO_2$ were grown in the standard way with 250 or 500 ml medium. In testing for the toxicity of amino acids, cultures were grown in 20 ml screw-capped Kimax test tubes, or tubes sealed with Suba-Seal rubber vaccine caps, and completely filled with medium.

Measurement of Growth. Direct readings of the optical density (E_{600}) of cultures could be made by placing the tubes in a Coleman Junior II spectrophotometer or an EEL colorimeter. As growth was generally accompanied by sulphur precipitation during the first 20—40 hrs, these measurements were not direct measures of biomass (cf. van Gemerden, 1968), but served, together with the visual development of the typical green colour, as a comparative measure of the rate of development of cultures. Initial values of E_{600}, 0.03—0.04, rose to 0.3—0.36, due entirely to the bacteria, after exhaustion of the sulphur, in 60—110 hrs in normal cultures. Growth was also evaluated by measuring culture absorbance at 745 and 665 nm, which gave a measure of pigment content as these values are the peak and trough of the absorption spectrum of the intact cells. Standard volumes (usually 5 ml) of cultures were also centrifuged, the bacterial chlorophyll extracted with 80% (v/v) methanol (40°C, 30 min) and E_{660} (the absorption maximum in methanol) measured to give an assessment of growth in terms of increase in pigment. Absorption spectra were run on Cary 14 and Unicam SP800 recording spectrophotometers. Increase in bacterial carbon was monitored with ^{14}C-bicarbonate by filtering culture samples through Millipore membranes (0.45 μm) and measuring fixed ^{14}C. Sulphur compounds in the cultures were determined by iodometric titration: samples were titrated for thiosulphate + sulphide in dilute acetic acid (Roy and Trudinger, 1970) and for thiosulphate after removal of sulphide by precipitation with cadmium. Sulphite was not detected in any cultures, so treatment with formaldehyde was unnecessary.

Isotope Procedures. Methods for the fractionation, hydrolysis, chromatography, radioautography and counting of labelled bacteria have been described previously (Kelly, 1967, 1969a, 1970; Eccleston and Kelly, 1973a). Most of the 660 nm-absorbing pigment was present in the 70% ethanol fraction (Kelly, 1967).

Preparation of Cell-Free Extracts and Assay of Aspartokinase. Cultures (51) were grown with constant illumination and slow magnetic stirring to the stationary phase. After harvesting at 3°C and suspending in 0.02 M potassium phosphate pH 7.0 containing 0.03 M mercaptoethanol, cell suspensions were passed once or twice through a French pressure cell at 3°C and 20000 lb/in^2. No anaerobic procedures were used

at this stage, and the extracts were then centrifuged at 30000 × *g* for 30 min at 3° C. The dark green supernatant liquid contained 10—20 mg protein per ml and was used either directly for enzyme assay or after overnight dialysis against the buffer solution at 3° C.

Aspartokinase (EC 2.7.2.4) was assayed (Eccleston and Kelly, 1973b) with the following reaction mixture (μmoles/ml): hydroxylamine hydrochloride, 800; potassium hydroxide, 750; Tris-HCl pH 8.0, 100; $MgCl_2$, 10; ATP, 10; mercaptoethanol, 10; aspartate, 10; extract protein, 1—2 mg. After incubation at 30° C for up to 60 min, 1 ml 10% (w/v) $FeCl_3$ in 3.3% (w/v) trichloroacetic acid containing 0.7 N HCl was added. The precipitate was centrifuged down and the E_{540} (1 cm) of the liquid measured. Absorbance due to aspartyl hydroxamate was linear relative to concentration up to 1.0 using a Unicam SP500 or Cary 14 spectrophotometer. In the standard assay procedure, an absorbance of 0.350 was obtained with 1 μmole of aspartyl hydroxamate (Sigma). Blanks without extract or without aspartate or with the $FeCl_3$ reagent added initially were also measured.

Results

Normal Development of Chlorobium Cultures. When grown on 4 mM sodium sulphide as sole electron donor, *Chlorobium* 8327 completely oxidized this in 48 hrs with an essentially quantitative accumulation of sulphur, which underwent only very slow further oxidation. This is commonly found with *Chlorobium* spp (van Niel, 1931). Cultures containing initially 4 mM thiosulphate and 0.6 mM sulphide showed a rapid drop in sulphide content and a progressive fall in the total titratable sulphur and in thiosulphate (Fig. 1). Commonly a visible accumulation of extracellular sulphur accompanied thiosulphate disappearance, measurable as a considerable increase in culture turbidity (Fig. 1a), which subsequently declined. Frequently this sulphur was essentially completely oxidized to sulphate. While direct absorbance measurements were of little value in the quantitative assessment of growth, they served, with the observation of "greening" as a very useful indication of the effects of toxic materials. Extraction into methanol of the pigment from standard samples of bacterial cultures showed that growth, as relative increase in extractable pigment (E_{660}) accompanied sulphur compound oxidation with the approximation of both to exponential growth kinetics (Fig. 1b) with mean generation times of the order of 5—9 hrs.

Growth measured as $^{14}CO_2$-fixation by growing cultures also showed an approximate parallel to thiosulphate oxidation (Table 1) although further growth (i.e. $^{14}CO_2$-fixation) could occur after the disappearance of iodine-titratable sulphur compounds from the medium. This was presumably at the expense of elemental sulphur: no sulphite was detected in the cultures. For growth inhibition and ^{14}C-assimilation experiments, test compounds were added either prior to medium inoculation or during growth. Samples were removed both when titratable thiosulphate was still present and when all precipitates of sulphur had gone. Measurements

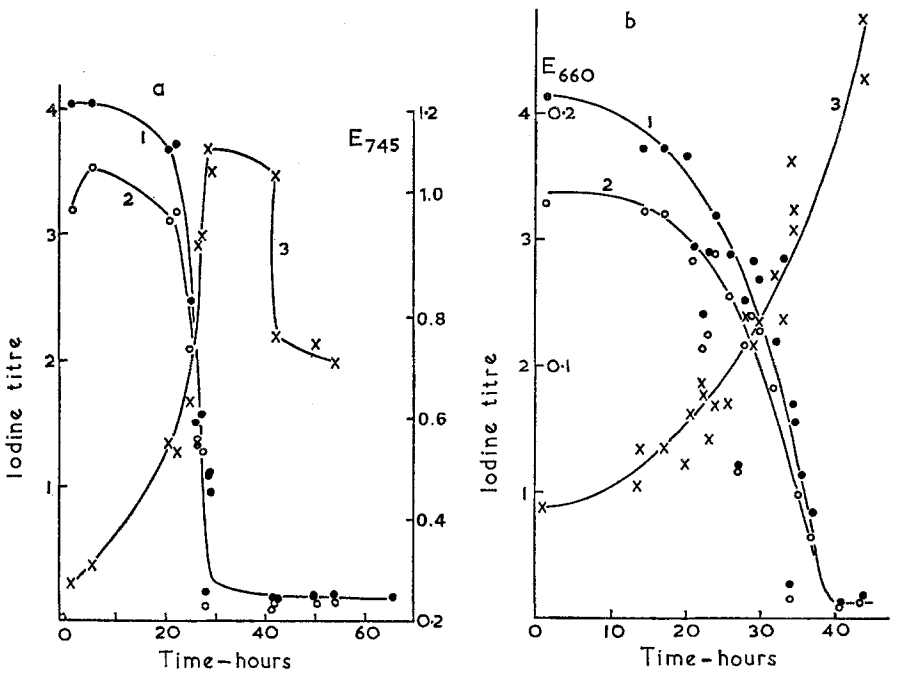

Fig. 1 a and b. Growth of *Chlorobium* 8327 in illuminated bottles on thiosulphate medium. (a) *Curve 1*, total titratable sulphur; *curve 2*, thiosulphate titre; *curve 3*, $E_{745\,nm}$ of whole culture suspension. (b) *Curve 1*, total titratable sulphur; *curve 2*, thiosulphate titre; *curve 3*, $E_{660\,nm}$ of pigment extracted into methanol

of the absorbance of cultures at the latter stage were indicative of biomass both when E_{600} and the E_{745}/E_{660} values were used.

Effect of Organic Nutrient Compounds on the Growth of Chlorobium. The growth of normal 20 ml cultures with 5% (v/v) inocula in sealed tubes generally commenced within 20 hrs and was complete in 80—130 hrs. Normal development was unaffected by the following compounds at 1 mM: L-aspartate, L-glutamate, L-alanine, L-arginine, L-valine, L-proline, L-hydroxyproline, L-leucine, L-isoleucine, L-histidine, L-citrulline, L-phenylalanine, D-phenylalanine, D-methionine, shikimic acid; or by 10 mM L-lysine, D-glucose, D-ribose; or by 1% (v/v) ethanol, propanol or isopropyl alcohol. Growth was stimulated by glucose and by acetate (cf. Hoare and Gibson, 1964). Several amino acids at 1 mM prevented growth commencement for periods between 7—15 days or prevented any growth at all. These were L-methionine, L-tyrosine, L-tryptophan, L-threonine, L-serine and glycine. Approximate minimum inhibitory concentrations (causing slight retardation of normal development) were 0.01 mM L-methionine, 0.01 mM glycine, 0.1 mM L-serine, 0.1 mM L-tryptophan,

Table 1. Growth of *Chlorobium* 8327 measured by titratable sulphur, $^{14}CO_2$-fixation and absorbance of culture suspensions at 745 nm

Time (hrs)	Titratable sulphur[a]		E_{745}	$^{14}CO_2$ fixed (dpm)
	Total[b]	Thiosulphate[c]		
0	4.04	2.92	—	10
15.5	1.74	1.17	0.093	7551
18.0	1.98	0.77	0.120	7544
20.0	1.85	1.48	0.134	9649
21.3	1.64	1.30	0.355	18883
27.5	1.47	1.17	0.450	20698
30.7	1.33	0.94	0.355	24152
36.0	0.98	0.78	0.450	32577
44.5	0.48	0.34	0.538	38041
62.5	0.14	0.09	0.565	50911

[a] ml 0.005N iodine/10 ml of culture.
[b] Sulphide + thiosulphate.
[c] Determined after precipitation of sulphide with Cd^{2+}.

0.01 mM L-tyrosine, 0.02 mM L-threonine. Frequently, 0.1 or 1 mM of these compounds appeared lethal or resulted in growth after such protracted periods (15—34 days) that selection of rare resistant mutants was presumed to have occurred.

Sodium formate also produced lags before growth began. The time taken for visible growth to develop and to be complete increased with formate concentration from 3 and 4—6 days respectively with control cultures to 6 and 9 days (10^{-5} M), 9 and 13 days (10^{-4} M), and 13 and 17 days (10^{-2} M).

Effect of Mixtures of Amino Acids on Growth. If growth inhibition by single amino acids was due to inhibition of early enzymes of branched biosynthetic pathways, resulting in starvation of the cell of essential amino acids (Kelly, 1969a, 1971; Rittenberg, 1969, 1972; Lu *et al.*, 1971), supplementation of the inhibitory media with other amino acids might overcome the growth inhibition. The results of several such experiments are summarized in Table 2. Inhibition by tyrosine and tryptophan, separately or in mixture, was overcome by phenylalanine, but not by 1 mM shikimate. Tryptophan (0.1 mM) inhibition could also be overcome by a mixture of leucine, isoleucine and alanine. Valine was sometimes toxic at 10 mM, and this effect was largely prevented by 1 mM isoleucine. The toxic effects of threonine and methionine was slightly affected by lysine, but could be completely prevented by isoleucine. Glycine and serine were toxic separately and in mixture. While some of these effects may be explained in terms of supply of amino acids whose synthesis was blocked

Table 2. Inhibitory effects of some amino acids alone and in mixture with other amino acids on the growth of *Chlorobium*

Treatment (mM)	Time taken (days) for growth	
	Commencement	Completion
Controls	1—2	4—6
a) Glycine (0.1)	37 +	—
Serine (0.1)	23 +	—
Gly + Ser	37 +	—
b) Threonine (0.1)	37 +	—
Methionine (0.1)	12	17
Thr + Met	37 +	—
Thr + Met + Lysine (1.0)	10	13
Thr + Met + Lys + Isoleucine (1.0)	1—2	4—6
c) Thr (0.1) + Met (0.05)	7 +	—
Thr + Met + Lys (1.0)	7 +	—
Thr + Met + Lys + Isoleucine (1.0)	1—2	3—4
d) Threonine (0.1)	23 +	33
Thr + Met (0.05)	33 +	—
Thr + Met + Lys (1.0)	33 +	—
Thr + Met (0.01)	33 +	—
Thr + Met + Lys	23	26
e) Threonine (1.0)	7 +	13
Thr + Lys (1.0)	7 +	13
Methionine (0.1)	7 +	13
Met + Lys	13 +	—
Met + Thr + Lys	7 +	13
Met + Thr + Lys + Ile (1.0)	2	4
f) Valine (10.0)	23 +	33
Val + Isoleucine (1.0)	6	9
Tryptophan (0.1)	2—3	7
Trp + Leu (1) + Ala (1) + Ile (1)	1—2	3
Trp (1.0) + Leu + Ala + Ile	33 +	—
g) Trp (0.5)	8 +	10—11
Tyrosine (0.5)	10 +	—
Tyr + Phenylalanine (1.0)	2	5—6
Tyr + Trp + Phe	2	5—6

Letters indicate separate experiments. For experiment e), a large actively growing inoculum was used.

by the inhibitory amino acids, an alternative explanation could be that uptake of the inhibitory amino acid was blocked by the non-toxic ones, as has been reported for *Thiobacillus* and *Methylococcus* (Kelly, 1969b; Eccleston and Kelly, 1972a, b).

Table 3. Depression by some other amino acids of the amount of ^{14}C-leucine incorporated by growing cultures of *Chlorobium*. Cultures (20 ml in Kimax tubes) contained 0.01 mM ^{14}C-leucine (566 cpm/nmole), other amino acids as indicated and a 4% (v/v) inoculum. Initial E_{600} = 0.032. When no more precipitated sulphur was visible (114—280 hrs) triplicate samples were filtered to measure ^{14}C-incorporation

Additions (mM)	Final E_{600}	^{14}C-Leucine incorporated	
		nmoles/ml	nmoles/0.1 increase in E_{600} during growth
Control (leu only)	0.243 ± 0.013	6.18 ± 0.09	2.55 ± 0.17
Isoleucine (1.0)	0.313	1.16	0.41
Phenylalanine (1.0)	0.321	1.43	0.49
Valine (1.0)	0.265	1.29	0.55
Tryptophan (0.1)	0.382	3.47	0.99
Methionine (0.1)	0.262	2.51	1.09
Alanine (1.0)	0.309	3.48	1.26
Glutamate (1.0)	0.318	6.54	2.29
Proline (1.0)	0.298	6.69	2.52
Arginine-HCl (1.0)	0.290	6.51	2.52
Hydroxyproline (1.0)	0.288	6.88	2.69
Aspartate (1.0)	0.291	6.21	2.40
Threonine (1.0)	0.258	5.81	2.34
Lysine-HCl (1.0)	0.308	6.51	2.36
Histidine (1.0)	0.218	5.60	3.01
Citrulline (1.0)	0.278	6.48	2.63

Addition of a further 1 mM unlabelled leucine to the control reduced ^{14}C-incorporation to 288 cpm/ml from 3500 cpm/ml, indicating *ca.* 50 nmoles leucine incorporated, or some 18.3 nmoles/0.1 increase in E_{600} due to growth. The concentration of leucine used was therefore not enough to saturate the transport system.

Cross-Inhibition of Uptake of Amino Acids from Mixtures. ^{14}C-labelled leucine was incorporated by growing cultures of *Chlorobium* 8327, but its assimilation was depressed by isoleucine, phenylalanine, valine, tryptophan, alanine and methionine (Table 3). The other amino acids tested had no significant effect. Similarly, assimilation of ^{14}C-threonine (0.05 mM) was depressed at least 80%, and growth inhibition relieved by isoleucine or phenylalanine (0.5 mM), but ^{14}C-aspartate (0.1 mM) assimilation was not significantly affected by threonine (0.1 mM) or lysine (1 mM).

Assimilation of ^{14}C-Labelled Substrates by Chlorobium Growing in Otherwise Normal Media. Glucose and a number of amino acids were quite readily assimilated, although little lysine (supplied at a rather low concentration) was taken up. Incorporation of L-phenylalanine was little affected by D-phenylalanine (Table 4). Sodium ^{14}C-formate was also incorporated. Cultures grown on ^{14}C-labelled carbon dioxide, formate or

Table 4. Ability of *Chlorobium* 8327 to incorporate various ^{14}C-labelled substrates during photolithotrophic growth

Addition to normal medium	Concentration (μmoles/l)	Growth period (hrs)	^{14}C-compound consumed (μmoles/l)	Growth (δE_{600})
^{14}C-Phe	100	120	26.0	0.327
^{14}C-Phe	500	120	35.0	0.286
^{14}C-Phe + ^{12}C-D-Phe	100 + 400	120	28.4	0.275
^{14}C-Leucine	100	120	65.1	0.355
^{14}C-Tyrosine	100	432[a]	16.6	0.278
^{14}C-Glucose	34	120	14.8	0.274
^{14}C-Glucose	143	120	29.7	0.297
^{14}C-Aspartate	0.42	4	0.30	—
^{14}C-Lysine	8.3	3	0.05	—
		20	0.10	—
^{14}C-Homoserine	67	3	3.50	—
		20	16.50	—
^{14}C-Threonine	8.3	3	0.20	—
		20	1.34	—

[a] Normal growth delayed 260 + hrs by the tyrosine.
— = not measured.

amino acids (with and without additional unlabelled amino acids) were analyzed in detail to see how extensively these exogenous materials were metabolized, and to see if information on biosynthetic pathways and regulatory mechanisms could be obtained (Table 5). Carbon from some of these compounds entered all the major cell fractions, although threonine and homoserine were preferentially incorporated into protein, and formate into the nucleic acid fraction (soluble in hot 5% TCA). Threonine, lysine or homoserine did not greatly affect the labelling distribution from ^{14}C-aspartate; very little lysine was metabolized; and the incorporation of threonine was very severely depressed by isoleucine (Table 5).

Hydrolysis of the nucleic acid material after uptake of ^{14}C-formate showed virtually all the ^{14}C to have been converted to purines (Table 6). At least 77% of the formate-carbon incorporated was thus recovered in only two compounds from the *Chlorobium*.

Analysis of the hydrolysed protein fractions from *Chlorobium* grown with various ^{14}C-labelled additives enabled some of its metabolic mechanisms to be tentatively identified. ^{14}C-labelled lysine or phenylalanine or tyrosine or leucine were incorporated unchanged predominantly into the protein of the cells. No mechanisms thus operated for their further transformation or interconversion. ^{14}C from glucose or formate appeared in all the components of the protein, as of course did $^{14}CO_2$, the major carbon

Table 5. Distribution among cell fractions of ^{14}C from various compounds incorporated by growing cultures of *Chlorobium* 8327. Organisms from 20 or 50 ml cultures were suspended into 5 ml for analysis

Additions to medium	Incubation time	Distribution (%) of ^{14}C in fractions					Recovery of total ^{14}C (% of that in initial labelled bacteria)	Total ^{14}C taken up
		Cold 5% TCA	70% Ethanol (Lipid + Pigment)	Ether + Ethanol	Hot 5% TCA	Residue (Protein)		
(μM)	(hrs)							(dpm/ml)
$^{14}CO_2$	4—20	3.8	10.6	10.2	17.6	57.8	98—112	$0.7—2.4\times10^6$
^{14}C-Asp (0.42)	4	4.5	12.8	9.1	20.4	53.2	100.0	1.1×10^6
^{14}C-Asp + 100 Lys	4	4.1	15.7	6.2	19.6	54.4	99.9	1.1×10^6
^{14}C-Asp + 100 Homoserine	4	5.8	19.9	9.3	19.0	46.0	77.5	1.4×10^6
^{14}C-Asp (100)	166	4.3	12.0	9.1	15.5	59.1	98.6	1.1×10^6
^{14}C-Asp + 100 Thr	360	3.8	9.5	12.9	14.5	59.3	95.6	1.1×10^6
^{14}C-Lysine (8.3)	3	69.8	2.7	0.2	4.4	22.9	106.5	14140
	20	71.7	2.2	1.2	3.5	21.4	85.9	25725
^{14}C-Homoserine (67)	3	11.1	5.6	0.3	5.1	77.9	91.5	27860
	20	22.1	1.5	0.6	2.1	73.9	115.5	163545
^{14}C-Thr (8.3)	3	18.9	6.2	0.5	3.0	71.4	94.1	55539
	20	16.0	2.1	0.8	1.7	73.7	107.7	549172
^{14}C-Thr + 100 Ile	3	56.5	6.5	0.5	6.2	30.3	90.6	19394
	20	32.7	2.9	3.1	5.0	56.3	111.2	42938
^{14}C-Serine (50)	[a]	3.1	17.0	2.2	18.8	58.9	94.9	1.55×10^6
^{14}C-Formate (100)	216	1.4	10.6	1.1	79.6	7.3	96.4	220000

[a] 34 day lag preceded growth.

source (Table 7). Carbon from aspartate appeared in all the amino acids, but compared with the normal protein-carbon distribution (i.e. the $^{14}CO_2$-label pattern) proportionately more aspartate-carbon was incorporated into aspartate, threonine, methionine and lysine than into other amino acids. Aspartate conversion to lysine or to threonine was slightly depressed by unlabelled lysine or by unlabelled threonine or homoserine (Table 7). Labelling from ^{14}C-homoserine appeared exclusively in threonine, methionine and isoleucine (confirmed by separate chromatography

Table 6. Distribution of ^{14}C from formate among the components of the nucleic acid fraction (soluble in hot 5% trichloroacetic acid). After hydrolysis with HCl, bases were separated by two-dimensional ascending paper chromatography using isopropanol-HCl and butanol-ammonia solvents (Smith, 1960)

Component	^{14}C content	
	cpm/chromatogram spot	% of total
Adenine	9777	59.0
Guanine	6250	37.7
Uridylic acid	382	1.0
Cytidylic acid	165	2.3

Table 7. Distribution of ^{14}C among amino acids in the hydrolysed proteins

Amino acid	^{14}C-content (% of total in protein) after assimilation of				
	$^{14}CO_2$[a]	H^{14}COONa	^{14}C-Asp	^{14}C-Asp + ^{12}C-Lys	^{14}C-Asp + ^{12}C-Homoserine
Asp	9.43 ± 0.15	4.83	**16.21**	16.24	16.99
Glu	13.78 ± 0.52	7.79	11.74	10.88	12.02
Gly	7.51 ± 0.20	0.85	4.33	4.46	4.74
Ser		1.40			
Thr	4.62 ± 0.18	2.71	**7.99**	7.87	**4.49**
Ala	7.42 ± 0.12	5.25	5.56	5.66	6.07
Tyr	4.93 ± 0.21	9.14	3.74	4.06	4.10
Val	7.45 ± 0.23	5.37	**7.00**	7.04	6.33
Met					
Met SO	1.28 ± 0.30	3.51	**2.65**	2.82	2.61
Phe	23.04 ± 0.60	13.63	20.56	21.31	22.34
Leu		7.49			
Ile		8.84			
Pro	4.95 ± 0.15	5.37	3.84	4.09	4.08
Arg	5.83 ± 0.38	7.41	3.74	3.73	3.59
Lys	9.82 ± 0.37	9.61	**12.64**	**11.84**	12.63
Cys	—	6.82	—	—	—

[a] Mean ± range for three separate experiments with 4—20 hrs exposure to $^{14}CO_2$.

of the leucine-isoleucine-phenylalanine mixture from hydrolysates). Most ^{14}C-threonine appeared unchanged in protein, with a small amount of conversion to isoleucine. Serine appeared in protein largely as unchanged serine, but was partly converted to glycine, alanine, cysteine and aspartate (Table 7).

Aspartokinase Activity in Chlorobium Extracts and Its Sensitivity to Amino Acids of the Aspartate Family. Crude extracts showed aspartokinase activity when assayed by standard aerobic procedures. Absorption spectra of the reaction mixtures + $FeCl_3$ reagent showed single peaks at 530 nm due to the aspartyl hydroxamate product. Optimum pH for assay was pH 8.0 (Fig. 2a). When undialysed extracts were used a high blank value was obtained in the absence of added aspartate. This could be 20—25% of the control value or occasionally even higher (Fig. 2b; cf. Eccleston and Kelly, 1973b), but was apparently due largely to aspartate already present in the extracts as it (a) was decreased by dialysis; (b) ceased after a finite time (Fig. 2b); and (c) showed the same inhibition properties as the control reaction. Aspartokinase activity was strongly inhibited by L-threonine or L-lysine, and more strongly by both together (Table 8 and Fig. 2b). In another experiment 0.09 mM L-threonine inhibited by 24%, 0.9 mM L-lysine by 35%, and the pair by 71%.

of *Chlorobium* after the assimilation of various labelled compounds

^{14}C-Homoserine	^{14}C-Thr	^{14}C-Thr + ^{12}C-Ile	^{14}C-Ser	100 µM ^{14}C-Asp	^{14}C-Asp + 100 Thr	^{14}C-Asp + 1000 Lys
—	—	—	3.90	13.3	12.5	13.0
—	—	—	—	11.1	11.4	11.9
—	—	—	26.60	5.7	5.9	5.4
—	—	—	57.30			
66.44	97.35	92.40	—	6.3	4.6	6.1
—	—	—	5.50	7.4	7.8	7.5
—	—	—	—	5.4	5.6	5.7
—	—	—	—	7.5	6.6	7.6
12.64	—	—	—			
15.49	—	—	—			
6.43	—	—	—	22.1	23.3	22.9
	—	—	—			
	2.65	7.60	—			
—	—	—	—	4.4	4.7	5.0
—	—	—	—	6.2	6.0	5.8
—	—	—	—	10.7	11.6	9.1
—	—	—	6.70	—	—	—

For details of culture conditions see Table 5.

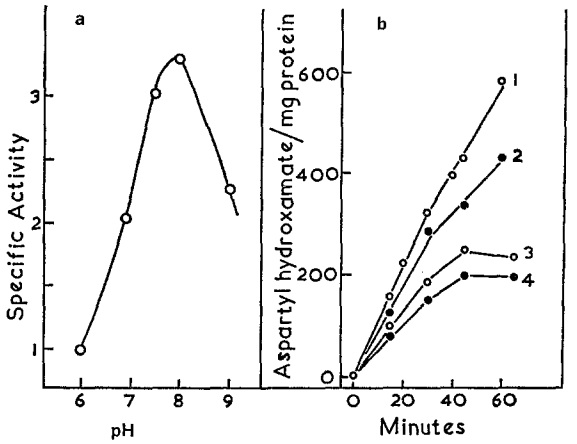

Fig. 2a and b. Aspartokinase activity in crude extracts of *Chlorobium*. (a) Specific activity (nmoles aspartyl hydroxamate formed min^{-1} mg^{-1}) at various pH values, determined from 60 min incubations with 1.5 mg protein per assay. (b) Aspartyl hydroxamate formation by aspartokinase in undialysed crude extracts in the presence or absence of L-threonine. *Curve 1*, control with aspartate; *curve 2*, + aspartate with 0.01 mM L-threonine; *curve 3*, no added aspartate; *curve 4*, — aspartate with 0.01 mM L-threonine. Each assay used 1.5 mg crude extract protein; control specific activity was *ca.* 9.7 nmoles aspartyl hydroxamate min^{-1} mg^{-1}

Table 8. Effect of aspartate family amino acids on the activity of aspartokinase in crude extracts of *Chlorobium* 8327

Addition to assay mixture	Concentration (mM)	Activity[a]	Inhibition (%)
None (control)	—	10.4	0
L-Threonine	0.01	7.0	33
	0.1	2.2	79
	1.0	0	100
	10.0	0	100
L-Lysine	0.1	4.5	57
	1.0	1.2	88
	10.0	1.0	90
L-Thr + L-Lys	0.01 + 0.1	0	100
D-Threonine	1.0	7.0	33
L-Methionine	10.0	10.0	4
L-Isoleucine	10.0	10.2	2
L-Met + L-Ile	10.0	11.7	(+ 16)

[a] nmoles aspartyl hydroxamate/mg protein/min at 30° C.

D-Threonine was also slightly inhibitory, though no more so than would have resulted from a 1% (w/w) content of L-threonine in it (Table 8). Methionine and isoleucine were without significant effect on activity.

Discussion

Although studies on the physiology of *Chlorobium* have been under way for many years (Nadson, 1912; van Niel, 1931; Larsen, 1953), it is only recently that detailed information on its biochemistry has been obtained (Evans *et al.*, 1966; Sirevåg and Ormerod, 1970; Trüper and Peck, 1970; Kusai and Yamanaka, 1973). Little work has been done on intermediary carbon metabolism, and even the main mechanism of carbon dioxide fixation is in dispute (Pfennig, 1967; Kelly, 1971; Beuscher and Gottschalk, 1972; Buchanan *et al.*, 1972; Sirevåg, 1974). Few studies have been made on the growth rates and yields of batch cultures on sulphur compounds (Larsen, 1953; Shaposhnikov *et al.*, 1958), although several workers have demonstrated that some organic compounds including acetate, pyruvate and propionate can be metabolized and may stimulate growth (Larsen, 1953; Sadler and Stanier, 1960; Hoare and Gibson, 1964; Nesterov *et al.*, 1965; Pfennig and Lippert, 1966). In the experiments reported here the progress of cultures could be monitored by titration, carbon fixation and increase in pigment, and indicated a generation time under the conditions used of 5—9 hrs.

There seem to have been few studies on the toxicity or assimilation of more complex organic compounds by *Chlorobium* and it has been suggested that their cells are impermeable "to the majority of organic compounds" (Evans and Whatley, 1970). This view is clearly not borne out by these and other recent experiments. The only other work with amino acids seems to be that of Belousova (1968) who showed *Chlorobium* to use proline or asparagine as nitrogen sources and to be stimulated by norleucine, glutamate and arginine.

A number of conclusions can be drawn from the experiments I have reported:

1. Numerous organic compounds can be assimilated by photolithotrophic cultures, although these compounds alone do not support growth.

2. The patterns of labelling among cell constituents after assimilation of ^{14}C-labelled substrates indicate that the pathways of intermediary metabolism for these compounds in *Chlorobium* are similar to those known in many other organisms (see Fig. 3 for scheme).

3. Formate assimilation is largely into purines and indicates little equilibrium exchange with carbon dioxide.

4. A number of amino acids and formate can be growth inhibitory. The mechanism for inhibition by most of these has not been established,

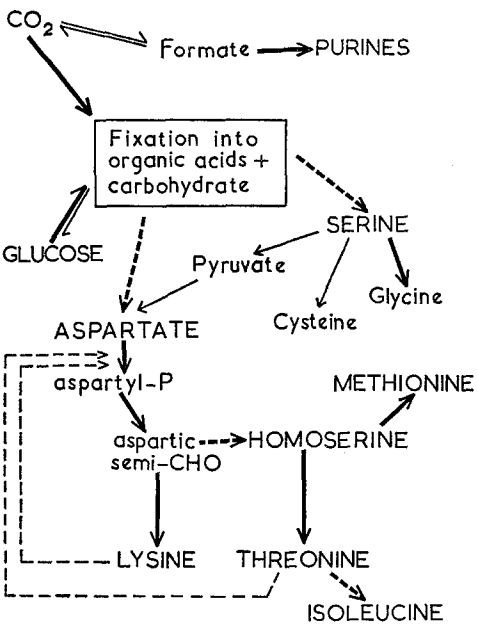

Fig. 3. Biosynthetic routes in *Chlorobium* as indicated by the experiments on ^{14}C-labelling and enzyme assay. ——→, Directly indicated pathways; - - - →, presumed major pathways; ——→, lesser activity pathways; - - - →, end product inhibition

although they will probably prove to be due to "autostarvation" resulting from the inhibition of primary enzymes of branched pathways.

5. Aspartokinase is under inhibition control by the endproducts of the aspartate pathway: lysine and threonine, both of which can inhibit it completely, as well as showing a cooperative effect. Aspartokinase inhibition is probably the basis for threonine toxicity. Lysine is not growth-inhibitory, but this can be explained because lysine is taken up by the organisms only to a very small extent.

6. Amino acid uptake is probably brought about by several systems, each being used for a number of amino acids. It is not known if specific amino acid permeases exist. These low-specificity general transport systems, which occur in other lithotrophic and methylotrophic bacteria show competition for uptake between the various compounds transported (Kelly, 1969b, 1971; Eccleston and Kelly, 1972b).

7. The presence of externally added amino acids did not result (under conditions where growth was still possible) in the cessation of internal synthesis of those amino acids, indicating that for the acids tested repression or complete inhibition of endogenous synthesis was not possible, at least under the conditions of the experiments.

One can conclude that the unique features of *Chlorobium* reside in its obligately anaerobic photolithotrophy (and possibly its primary carbon dioxide fixation mechanism), but that its intermediary biosynthesis and its control of pathways by end-product inhibition are probably similar in general to mechanisms well known to comparative biochemistry. In natural environments the organism probably shows a photomixotrophic carbon nutrition, and obtains significant quantities of its carbon and nitrogen from the photoassimilation of organic compounds.

Acknowledgements. I wish to thank Nadia El Borai, G. H. Owen and P. C. Poole, who have carried out some experiments on *Chlorobium* for this study; to Olga Molnár, Irene Verners and Esther Choolun for technical assistance; to the Baas Becking Geobiological Laboratory, Canberra, in which some of the work was carried out; and to June Lascelles and Norbert Pfennig to whom I am indebted for some insights into the mysteries of the green sulphur bacteria.

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