The Assimilation of Carbon by *Chloropseudomonas ethylicum*

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1. The enzymes in ultrasonically prepared extracts of *Chloropseudomonas ethylicum* were studied to elucidate how this organism assimilates acetate and carbon dioxide and why it cannot grow with either of these two compounds alone. 2. Such extracts can (i) convert acetate and oxaloacetate into α -oxoglutarate, (ii) convert oxaloacetate into succinyl-CoA, (iii) convert phosphopyruvate into 3-phosphoglyceraldehyde and (iv) interconvert phosphopyruvate and pyruvate via oxaloacetate. 3. Pyruvate kinase, α -oxoglutarate dehydrogenase, ribulose diphosphate carboxylase, isocitrate lyase and malate synthase were not detected. 4. It is difficult to detect aconitate hydratase, fumarate hydratase and citrate synthase in extracts of the organism ultrasonically treated in tris buffer; to demonstrate these enzymes extracts should be prepared in phosphate buffer containing 2-mercaptoethanol. 5. Provided that this organism can synthesize pyruvate from acetate and carbon dioxide, the enzymes detected are sufficient to account for the nutritional requirements of this organism.

Chloropseudomonas ethylicum (Shaposhnikov, Kondrat'eva & Fedorov, 1960) is a green obligate photosynthetic anaerobic bacterium. Unlike *Chlorobium* sp., which can be grown autotrophically, *Chloropseudomonas* cannot; it has an obligate nutritional requirement for carbon dioxide and a source of a C₂ unit best met in culture media by the addition of either ethanol or acetate.

To find out why *Chloropseudomonas* has this particular nutritional requirement, a study of the enzymes that could be concerned with its assimilation of carbon compounds was undertaken. This paper reports the results of this investigation; a preliminary account has already been published (Callely & Fuller, 1967). Smillie & Evans (1963) have already reported the presence of some tri-carboxylic acid-cycle enzymes in *Chloropseudomonas*; their extracts were prepared in tris buffer with a French pressure cell. The present paper also shows that for the measurement of some enzyme activities preparation of extracts in tris is to be avoided.

METHODS AND MATERIALS

Organisms. *Chloropseudomonas ethylicum* (strain 2K) was grown anaerobically at 30° in the light (light-intensity 200–250 ft.-candles) in screw-capped 1 l. prescription bottles completely filled with liquid medium. The medium was as

described by Bose (1963) except that acetate (final concn. 34 mM) was used instead of ethanol and the medium was additionally buffered with tris (final concn. 10 mM, pH 7.3). The inoculum was 20 ml. of a 2–3-day culture grown in the same medium. Samples of all inocula were added to test tubes two-thirds full of nutrient broth; absence of growth in these tubes in the dark and in the light during growth of the main culture was taken as a criterion of purity of the inocula. Cultures in the exponential-growth phase were harvested after about 22 hr. growth (cell density 220–250 mg. dry wt. of cells/l. of medium) by centrifuging at 3° for 25 min. at 8000g. Cells were washed twice by resuspension in either 50 mM-phosphate buffer or 50 mM-tris buffer (both pH 7.4) and centrifuged as above.

For confirmation of enzyme assay procedures when no activity could be demonstrated with the *Chloropseudomonas* extracts, extracts of either *Chromatium* (strain D) grown heterotrophically with acetate, or *Rhodospirillum rubrum*, both cultured as described by Bose (1963), were used.

Preparation of extracts. Extracts were prepared by treatment with a MSE-Mullard ultrasonic disintegrator for 3 min. at 0–5°. For ultrasonic treatment washed cells from 1 l. of medium were suspended in 2.5 ml. of buffer; centrifuging the ultrasonically treated material at 3° for 10 min. at 2000g was sufficient to sediment out sulphur and any remaining unbroken cells. The resulting supernatants (10–15 mg. of protein/ml.) were used in the various enzyme assays.

Boiled extracts were prepared by placing these supernatants in a boiling-water bath for 5 min.

Enzyme assays. Except for the acetate-activating system, measurements of the specific activities of enzymes were

Table 1. *Enzyme assay methods (spectrophotometric)*

Enzyme (a)	Substrate(s) (b)	Other additions (c)	Reference cuvette does not contain (d)	Wavelength used and molecular extinction coefficient (e)	Reference (f)
Aconitate hydratase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3]	Citrate (30 mm) or DL-isocitrate (10 mm)	Phosphate buffer (50 mm) or glycylglycine buffer, pH 7.4 (50 mm); extract	Citrate or isocitrate	240 m μ 3.54 $\times$ 10 ³	Racker (1950)
Citrate synthase [citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7]	Acetyl-CoA (about 0.1 mm); oxaloacetate (1 mm)	Glycylglycine buffer, pH 7.2 (50 mm); MgCl ₂ (5 mm); extract	Acetyl-CoA or oxaloacetate	232 m μ 4.5 $\times$ 10 ³	Dixon & Kornberg (1959)
Fumarate hydratase (L-malate hydro-lyase, EC 4.2.1.2)	Fumarate (17 mm)	Phosphate buffer (33 mm); extract	Fumarate	300 m μ 36.5	Massey (1955)
Isocitrate dehydrogenase (NADP ⁺ -linked) [<i>threo</i> -D-isocitrate-NADP ⁺ oxidoreductase (decarboxylating), EC 1.1.1.42]	DL-Isocitrate (0.2 mm)	Glycylglycine buffer, pH 7.4 (25 mm); NADP ⁺ (90 μ M); MnCl ₂ (0.6 mm); extract	NADP ⁺	340 m μ 6.22 $\times$ 10 ³	Ochoa (1955a)
Isocitrate lyase (<i>threo</i> -D-isocitrate glyoxylate-lyase, EC 4.1.3.1)	DL-Isocitrate (1.67 mm)	Glycylglycine buffer, pH 7.4 (33 mm); MgCl ₂ (5 mm); phenylhydrazine (4.2 mm); cysteine (2 mm); extract	Isocitrate	324 m μ 1.7 $\times$ 10 ⁴	Dixon & Kornberg (1959)
Malate dehydrogenase (L-malate-NAD ⁺ oxidoreductase, EC 1.1.1.37)	Oxaloacetate (0.25 mm)	Glycylglycine buffer, pH 7.4 (25 mm); NADH (0.1 mm); extract	NADH	340 m μ 6.22 $\times$ 10 ³	Ochoa (1955b)
Malate dehydrogenase (decarboxylating) (NADP ⁺ -linked) [L-malate-NADP ⁺ oxidoreductase (decarboxylating), EC 1.1.1.40]	L-Malate (0.5 mm)	Glycylglycine buffer, pH 7.4 (25 mm); NADP ⁺ (90 μ M); MnCl ₂ (1 mm); extract	NADP ⁺	340 m μ 6.22 $\times$ 10 ³	Ochoa (1955c)
Malate synthase [L-malate glyoxylate-lyase (CoA-acetylating), EC 4.1.3.2]	Acetyl-CoA (about 0.1 mm); glyoxylate (2 mm)	Glycylglycine buffer, pH 7.2 (50 mm); MgCl ₂ (5 mm); extract	Acetyl-CoA	232 m μ 4.5 $\times$ 10 ³	Dixon & Kornberg (1959)
α -Oxoglutarate oxidase system	α -Oxoglutarate (8.3 mm)	Phosphate buffer (33 mm); NAD ⁺ (90 μ M); cysteine (2.7 mm); MgCl ₂ (3 mm); thiamine pyrophosphate (0.1 mm); CoA (0.043 mm); extract	CoA	340 m μ 6.22 $\times$ 10 ³	Kaufman (1955)
Phosphopyruvate hydratase (enolase) (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11)	2-Phospho-D-glycerate (1.67 mm)	Tris buffer (16.7 mm); MgCl ₂ (1 mm); EDTA (10 μ M); extract	2-Phospho-D-glycerate	240 m μ 1.7 $\times$ 10 ³	Westhead & McLain (1964)

Pyruvate kinase (ATP-pyruvate phosphotransferase, EC 2.7.1.40)	Phosphopyruvate (1.67 mm) ADP (1.67 mm)	Tris buffer (16.7 mm); NADH ⁺ (0.15 mm); KCl (25 mm); MgCl ₂ (25 mm); lactate dehydrogenase (25 μg.); extract	NADH ⁺	340 mμ 6.22 × 10 ³	Bücher & Pfeiderer (1955)
Succinate dehydrogenase [succinate-(acceptor) oxidoreductase, EC 1.3.99.1]	Succinate (6.67 mm)	Phosphate buffer (16.7 mm); phenazine methosulphate (16.5 μg.); cytochrome c (1 mg.); extract	Succinate	550 mμ 2.10 × 10 ⁴	Massey (1959)
Succinyl-CoA synthetase [succinate-CoA ligase (ADP), EC 6.2.1.5]	Succinate (16.7 mm)	Tris buffer (33.3 mm); MgCl ₂ (3.3 mm); ATP (33 μm); CoA (33 μm); extract	Succinate	235 mμ 4.0 × 10 ³	First method listed by Cha & Parks (1964)
Triose phosphate dehydrogenase, [D-glyceraldehyde 3-phosphate-NAD oxidoreductase (phosphorylating?)]	3-Phospho-D-glycerate (45 mm)	Tris buffer, pH 8.0 (33.3 mm); MgSO ₄ (6.67 mm); cysteine (4 mm); ATP (0.92 mm); NADH (0.27 mm; reference cuvette contains half this); 3-phosphoglycerate (phospho)kinase (0.1 mg.); extract	3-Phospho-D-glycerate	340 mμ 6.22 × 10 ³	Heber, Pon & Heber (1963)

made by continually recording the progress of the reaction (at room temperature 18–22°) in quartz cuvettes (1 cm. light-path) in a Cary model 14 recording spectrophotometer. A recording of at least 1 min. preceded the addition of the substance that started the reaction and the recording was continued for at least 2.5 min.; enzyme activities were calculated from the initial rates of the reactions. The enzymes assayed spectrophotometrically and the methods used are listed in Table 1; the methods are essentially the same as described in the references in Table 1; any slight modifications introduced were minor. No attempt was made to determine optimum conditions for each assay.

The interconversion of 3- and 2-phosphoglycerate (either EC 2.7.5.3 or EC 5.4.2.1) was measured by substituting 3-phospho-D-glycerate for 2-phospho-D-glycerate in the phosphopyruvate hydratase (enolase) assay, adding excess of enolase (0.05 mg.) and recording the increase in E_{240} due to the production of phosphopyruvate.

Ribulose 1,5-diphosphate carboxylase (EC 4.1.1.39) activity was measured by the one-step method described by Racker (1962) and the two-step method described by Lascelles (1960).

The acetate-activating system was measured at 30° as described by Jones & Lipmann (1955); the extinction of the final colours produced was measured (as were all other individual extinction measurements) with a Zeiss PMQII spectrophotometer.

Protein determination. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), except that measurements were carried out at 850 mμ; this was because of interference by pigments in extracts of *Chloropseudomonas* that absorb strongly at 750 mμ (the recommended wavelength for this method) but not above 820 mμ.

Radioactive experiments. Conventional Warburg flasks contained (in the main compartment) 100 μmoles of tris, 10 μmoles of NaHCO₃ and 2 μmoles each of the other various substrates and cofactors; extract (3.1 mg. of protein) was placed in the side bulb. The flasks were attached to a Gilson differential respirometer (photosynthetic model GRP20) and the lamps switched on. The flask contents were equilibrated for 10 min. at 30° in an atmosphere of argon. Then 100 μC (2.5 μmoles) of NaH¹⁴CO₃ was added to the side bulb and together with the extract was immediately tipped into the main compartment; the total volume of reaction mixture was 1.0 ml. Any reaction was terminated after 10 min. by adding 4 ml. of ethanol and 1 ml. of acetic acid to the contents of each flask. A precipitate was removed by centrifuging and the resulting supernatants were gassed with argon for 45 min. to ensure complete removal of unused ¹⁴CO₂. A 0.1 ml. sample of supernatant was added to 15 ml. of scintillation fluid (Bray, 1960) and counted in a Nuclear-Chicago automatic scintillation counter. In the zero-time control the ethanol and acetic acid were added to the complete reaction mixture before the NaH¹⁴CO₃.

Chemicals. Unless otherwise stated the buffers used were at pH 7.4. Tris (final concn. 50 mm) was adjusted to the required pH with HCl; a solution of KH₂PO₄ (final concn. 50 mm) was adjusted to the required pH with NaOH.

Tris (Trizma base), NAD⁺, NADH, NADP⁺, ADP, ATP, IDP, cytochrome c, GSH, phenazine methosulphate, 3-phospho-D-glycerate (tricyclohexylammonium salt), 2-phospho-D-glycerate (barium salt, converted into a sodium

salt by dissolving in the minimal quantity of dilute HCl and adding a concentrated Na_2SO_4 solution until no further BaSO_4 was precipitated, removing the precipitate by centrifuging and adjusting the supernatant to pH 7.4 with NaOH, phosphopyruvate (trisodium salt), DL-isocitrate (trisodium salt), sodium glyoxylate and all the coupling enzymes were all the purest grade obtainable from Sigma Chemical Co. (St Louis, Mo., U.S.A.); acetyl-CoA and CoA were from C.F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). All other chemicals were of A.R. or the purest grade available.

$\text{NaH}^{14}\text{CO}_3$ was made by trapping, in NaOH, $^{14}\text{CO}_2$ that had been generated by the addition of acid to $\text{Ba}^{14}\text{CO}_3$ (40 mc/m-mole) (New England Nuclear Corp., Boston, Mass., U.S.A.).

RESULTS

Isocitrate dehydrogenase (NADP-linked) and malate dehydrogenase (NAD-linked) could readily be detected in extracts prepared by ultrasonic treatment of the organism in tris buffer. Aconitate hydratase activity in such extracts was, however, very difficult to demonstrate; only a trace amount of this enzyme could be detected and this infrequently. These difficulties were resolved when it was found that ultrasonic treatment of cells in tris buffer results in certain enzymes having a greatly lowered specific activity, and that adding 2-mercaptoethanol to the suspension before the ultrasonic treatment is beneficial.

Table 2 illustrates the variable activity of extracts prepared by ultrasonic treatment of the organism under different conditions. In such an experiment cells from four 11. cultures were pooled and then divided into two equal portions; one portion was washed twice with phosphate buffer, the other portion twice with tris. About half of the phosphate buffer-washed cells were resuspended in 2.5 ml. of the same buffer and ultrasonically treated as described above; the remainder were resuspended

in the same volume of buffer containing 2-mercaptoethanol (20 mm) and then ultrasonically treated. The tris-washed organisms were treated similarly, about half being ultrasonically treated after resuspension in 2.5 ml. of tris buffer, the remainder after resuspension in the same volume of buffer containing 2-mercaptoethanol (20 mm).

Aconitate hydratase was always assayed first and at about the same time (35–40 min.) after the start of the ultrasonic treatment of each batch of cells, as the specific activity of this enzyme in extracts falls rapidly during the first hours after preparation (Fig. 1a); for comparison the stability of fumarate hydratase is shown in Fig. 1(b).

Fig. 1. Stability of (a) aconitate hydratase and (b) fumarate hydratase in variously prepared extracts of *Chloropseudomonas*. The organism was ultrasonically treated and stored, at 0–5°, in: phosphate buffer plus 2-mercaptoethanol (●—●); phosphate buffer (●---●); tris buffer plus 2-mercaptoethanol (○—○); tris buffer (○---○). Experimental details are given in the text.

Table 2. *Specific activities of certain enzymes present in extracts of Chloropseudomonas prepared by ultrasonic treatment under different conditions*

Experimental details are given in the text. Organisms were treated ultrasonically in: T, tris buffer; TSH, tris plus 2-mercaptoethanol; P, phosphate buffer; PSH, phosphate plus 2-mercaptoethanol. The units of specific activity are μmoles/mg. of protein/hr.

		Specific activity			
		T	TSH	P	PSH
I	Aconitate hydratase	0.7	3.8	3.7	16.9
	Citrate synthase	15.8	16.9	117	120
	Fumarate hydratase	14	182	128	346
	Isocitrate dehydrogenase	165	124	158	151
	Malate dehydrogenase	327	235	314	289
	Succinate dehydrogenase	9.8	7.5	6.8	7.8
	Succinyl-CoA synthetase	2.4	2.7	2.5	3.1
	Phosphoglycerate (phospho)mutase	6.0	5.5	4.6	5.7
II	Phosphopyruvate hydratase	11.6	12.1	15.1	14.6

Fig. 2. Citrate synthase and related enzymes in extracts of *Chloropseudomonas*. Reaction cuvettes contained the usual cofactors and: in (a) acetyl-CoA and glyoxylate, extract (0.084 mg. of protein) being added at arrow X and oxaloacetate at Y; in (b) acetyl-CoA and pyruvate, extract (0.084 mg. of protein) being added at arrow X and oxaloacetate at Y. Control cuvettes lacked acetyl-CoA only; other experimental details are given in the text.

The specific activity of aconitate hydratase was confirmed by coupling this enzyme to added excess of isocitrate dehydrogenase and measuring the rate of reduction of NADP⁺ on the addition of citrate.

There was no activity if an equimolar amount of glyoxylate was substituted for oxaloacetate in the citrate synthase assay, indicating the absence of malate synthase (Fig. 2a); a very slow reaction was obtained if an equimolar amount of pyruvate was used as substrate, the rate being about one-hundredth of that with oxaloacetate (Fig. 2b).

Succinate dehydrogenase activity could readily be demonstrated with phenazine methosulphate as mediator; it made no difference whether phenazine methosulphate or extract was added to the cuvettes to start the reaction. In the assay for succinyl-CoA synthase, ATP was used and no attempt was made to see if GTP would replace it.

The only enzyme of the tricarboxylic acid cycle that has not been detected is 2-oxoglutarate dehydrogenase, though addition of an extract of *Rhodospirillum rubrum* (which contains this enzyme) to the same cuvettes in which no activity was being observed with the *Chloropseudomonas* extracts resulted in a rapid reduction of NAD⁺.

Extracts catalysed the production of (presumably) acetyl-CoA from acetate and CoA in the presence of ATP, Mg²⁺ ions and GSH, as indicated by the progressive formation of a compound that gave a positive hydroxamate reaction (Lipmann & Tuttle, 1945). The specific activity was 4.7 μmoles of product produced/mg. of protein/hr.

Isocitrate lyase could not be detected in any of

Table 3. Incorporation of ¹⁴C from H¹⁴CO₃⁻ into ethanol-soluble metabolites by cell-free extracts of *Chloropseudomonas*

Experimental details are given in the text. The additional compounds (each at 2 μmoles, including ATP and IDP) are listed below; the 'acetyl-CoA' was a mixture of 0.1 μmole each of acetate, ATP and CoA.

Supplements	Total activity in ethanol-soluble material (disintegrations/min./mg. of protein)
None	5480
Phosphopyruvate	164600
Phosphopyruvate + IDP	610200
Phosphopyruvate + ADP	118900
Pyruvate	652100
Pyruvate + ATP	923900
Pyruvate + 'acetyl-CoA'	3954500
Boiled extract and all supplements	Nil

the extracts, though rapid activity occurred when similarly prepared extracts of acetate-grown *Chromatium D*, known to contain this enzyme (Fuller, Smillie, Sisler & Kornberg, 1961), were added to the same cuvettes containing the *Chloropseudomonas* extract. This shows that the inability to demonstrate isocitrate lyase activity in *Chloropseudomonas* extracts was due neither to the presence of an inhibitor nor to a faulty assay procedure.

Reactions of the type:

were established with carbon dioxide as the source of C₁ and either pyruvate or phosphopyruvate as the C₃ compound. Extracts (prepared in tris) were incubated, as described above, with NaH¹⁴CO₃ and either phosphopyruvate or pyruvate in the presence and absence of appropriate cofactors; the amount of label incorporated into the ethanol-soluble metabolites is consistent with the presence of an IDP-dependent phosphopyruvate carboxykinase and a pyruvate carboxylase (Table 3). No 'malic' enzyme could be demonstrated; addition of extracts of *Chromatium D*, which contain this enzyme (Fuller *et al.* 1961), to the same cuvettes in which no activity was being observed with the *Chloropseudomonas* extracts resulted in a rapid reduction of NADP⁺. Carboxylation of ribulose 1,5-diphosphate could not be detected in extracts by either method, though activity was obtained when a *Rsp. rubrum* extract was added to the same cuvettes containing the inactive *Chloropseudomonas* extract.

Extracts contained phosphopyruvate hydratase; the specific activity of this enzyme was confirmed

by coupling to added pyruvate kinase and lactate dehydrogenase (both in excess) and measuring the rate of reduction of NAD^+ on the addition of 2-phosphoglycerate and ADP. By substituting 3-phosphoglycerate for 2-phosphoglycerate in the standard phosphopyruvate hydratase assay and adding an excess of this enzyme it was shown that extracts can catalyse the conversion of 3-phosphoglycerate into 2-phosphoglycerate, for no phosphopyruvate was produced from 3-phosphoglycerate, as indicated by an increase in E_{240} , until a *Chloropseudomonas* extract was added. No attempt was made to show any requirement for 2,3-diphosphoglycerate. The specific activities of these enzymes are shown in section II of Table 2.

Pyruvate kinase activity could not be detected though rapid activity was observed when pyruvate kinase (from rabbit skeletal muscle) was added to the same cuvettes containing the various *Chloropseudomonas* extracts.

Extracts also contained an active NAD-linked triose phosphate dehydrogenase, its specific activity being $33 \mu\text{moles/mg. of protein/hr.}$ The same rate of NADH^+ oxidation was obtained in this assay even if the added coupling enzyme phosphoglycerate (phospho)kinase (EC 2.7.2.3) was omitted from the reaction mixture.

DISCUSSION

While this work was in progress, Evans, Buchanan & Arnon (1966) proposed the reductive carboxylic acid cycle to account for the way by which the green photosynthetic bacterium *Chlorobium thiosulfatophilum* can assimilate carbon dioxide, which enters this cycle at four points, the reactions leading to a net synthesis of oxaloacetate. A number of reactions of this pathway are identical, with regard to the compounds involved, with those occurring in the tricarboxylic acid cycle, except that they are proceeding in the opposite direction. The cleavage of citrate leads to the regeneration of acetyl-CoA necessary for the ferredoxin-dependent carboxylation reaction that yields pyruvate. Such a cycle is unlikely to be present in *Chloropseudomonas* because of its obligate requirement for a C_2 compound and the consequent lack of necessity for the regeneration of a C_4 acceptor compound for carbon dioxide fixation.

A possible reason for the nutritional requirement of *Chloropseudomonas* would be that it lacks the citrate-cleavage enzyme and hence becomes dependent on an exogenous source of acetate to keep the pathway operative. If this were so it would be difficult to see what role aconitate hydratase was playing in this organism. Further, this organism possesses a citrate synthase.

It is always difficult to show that an enzyme is not

present in a cell-free extract because of inadvertent inactivation during the cell-breakage and extraction procedure. However, the absence of isocitrate lyase and malate synthase is consistent with the inability of the organism to grow with acetate as its sole carbon source. The absence of an α -oxoglutarate dehydrogenase is also no metabolic stumbling block, since the organism possesses more than enough of all the enzymes necessary to synthesize the carbon skeleton of glutamate from oxaloacetate and acetate, and also can synthesize succinyl-CoA (a precursor of chlorophyll) from oxaloacetate. Thus there is no need to think of these reactions as having to constitute a complete cycle (as in the tricarboxylic acid cycle) provided that the organism can readily synthesize oxaloacetate. These results indicate that *Chloropseudomonas* does this either by the acetyl-CoA-stimulated carboxylation of pyruvate or the IDP-dependent carboxylation of phosphopyruvate; only one of these reactions is theoretically necessary.

This leads to the problem of how pyruvate and phosphopyruvate are synthesized. Pyruvate kinase is not detectable and there is no evidence for a phosphopyruvate synthase. The data are consistent with the idea that these two C_3 compounds are interconvertible as follows:

But which gives rise to which?

There are two known possibilities. First, there is the carboxylation of ribulose 1,5-diphosphate to give 3-phosphoglycerate, the key reaction of the reductive pentose phosphate (Calvin) cycle (Bassham & Calvin, 1957). Certainly, extracts of *Chloropseudomonas* can catalyse the interconversion of 3-phosphoglycerate and phosphopyruvate, but ribulose diphosphate carboxylase has not been detected. Growth of some photosynthetic organisms with acetate is known to inhibit the synthesis of certain enzymes of the Calvin cycle, especially this carboxylase, e.g. in the alga *Chlamydomonas mundana* (Russell & Gibbs, 1966) and in the bacterium *Chromatium* D (Fuller *et al.* 1961); it is possible that in *Chloropseudomonas* this enzyme is permanently repressed and the metabolic price that *Chloropseudomonas* has to pay for its nutritional dependence on a C_2 compound is the absence of a functional Calvin cycle.

The other possibility is the production of pyruvate by the ferredoxin-dependent carboxylation of acetyl-CoA, which has been demonstrated in *Chlorobium thiosulfatophilum* (Evans *et al.* 1966) and in the non-photosynthetic anaerobe *Clostridium kluyveri* (Andrew & Morris, 1965). Firm confirmation of its presence in *Chloropseudomonas* awaits the

Scheme 1. Assimilation of carbon by *Chloropseudomonas ethylicum* grown with acetate and CO₂. Continuous arrows indicate definitely established reactions.

isolation of ferredoxin from this organism, but its presence would be consistent with the nutritional demand of *Chloropseudomonas* for carbon dioxide and a C₂ compound, the assimilation of which is summarized in Scheme 1.

The NAD-dependence of triose phosphate dehydrogenase is consistent with the phylogenetic distribution of this enzyme (see Fuller & Gibbs, 1959). That the same rate is obtained when the assay coupling enzyme (phosphoglycerate phosphokinase) is omitted from the reaction mixture means that extracts contain this enzyme as well or that the interconversion of glyceraldehyde 3-phosphate and 3-phosphoglycerate is direct, being catalysed by a NAD-dependent equivalent of EC 1.2.1.9 (Rosenberg & Arnon, 1955).

The specific activities of all the enzymes that catalyse reactions common to the tricarboxylic acid cycle are remarkably large; for example, they are all as large as or larger than those quoted for *E. coli* extracts (Gray, Wimpenny, Hughes & Mossman, 1966). Also noteworthy is the differing activity of extracts prepared by ultrasonic treatment of the organism in different buffers in the presence or absence of added thiol groups. The main variations are with citrate synthase, fumarate hydratase and aconitate hydratase.

The additional instability of aconitate hydratase increases the difficulty of its assay and detection in anaerobes. Thus Gottschalk & Barker (1966) found it necessary to preincubate extracts of *Clostridium kluyveri* (prepared by ultrasonic treatment in phosphate buffer plus mercaptoethanol) under hydrogen to obtain aconitate hydratase activity;

Stern & Bambers (1966), who prepared extracts of another *C. kluyveri* by ultrasonic treatment in tris plus cysteine with subsequent addition of mercaptoethanol, also point out that reducing conditions have a marked stabilizing or activating influence (or both) on aconitate hydratase.

Thus this enzyme in anaerobes would appear to be different to that in other organisms. In *Chloropseudomonas*, phosphate buffer (plus mercaptoethanol) must be used in preference to tris during the ultrasonic treatment, but it makes no difference which of these buffers is used during the assay procedure. This contrasts with the aconitate hydratases quoted by Dickman (1961) where the use of a phosphate buffer is to be avoided. The specific activities of aconitate hydratase and fumarate hydratase in extracts of *Chloropseudomonas* prepared in tris are similar to those quoted by Smillie & Evans (1963), who disrupted this organism in tris buffer with the aid of a French pressure cell; their inability to demonstrate aconitate hydratase activity in *Chlorobium thiosulfatophilum* could be due to the preparation of their extracts in tris.

Many of the enzymes associated with the tricarboxylic acid cycle are present and functioning in this obligate photosynthetic anaerobe. The primary purpose of these enzymes in this organism is not the reoxidation of nicotinamide nucleotides and the consequent production of ATP, but in their use as accessory carbon synthetic pathways for the rapid synthesis of cellular material. All the initial reactions necessary for synthesis are shown in Scheme 1; it would appear that this obligate photoheterotroph could undergo photosynthetic

growth by using the synthetic pathways described, and the absence of a Calvin cycle is reflected by the obligate nutritional requirement of a C₂ fragment.

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