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Glycollate Production and Excretion by *Alcaligenes eutrophus*

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Abstract. Autotrophic cultures of the facultative chemolithotroph *Alcaligenes eutrophus* have been found to excrete glycollate. This excretion was greatly stimulated by the incorporation of up to 20% (v/v) oxygen in the hydrogen used for gassing. The stimulatory effect of oxygen was prevented by the addition of 10% (v/v) CO_2 to the gassing mixture. Glycollate excretion only in the presence of oxygen was increased by the addition of 2-pyridyl-hydroxymethane sulphonic acid (HPMS), an inhibitor of glycollate oxidation, indicating that glycollate formation itself was stimulated by oxygen. No glycollate excretion by cultures grown heterotrophically on pyruvate was detected, either in the absence or presence of HPMS, under heterotrophic or autotrophic conditions.

Extracts from autotrophic cells showed phosphoglycollate phosphatase and glycollate oxidoreductase activities, which were considerably lower in extracts prepared from pyruvate- or fructose-grown (heterotrophic) cells. The increase in activity of both enzymes upon cell transfer from heterotrophic to autotrophic growth was prevented by chloramphenicol and resembled the induction of D-ribulose 1,5-diphosphate carboxylase under the same conditions.

Key words: Glycollate excretion – Phosphoglycollate phosphatase – Glycollate oxidoreductase – D-Ribulose 1,5-diphosphate carboxylase – *Alcaligenes eutrophus.*

The formation of glycollate during photosynthesis by higher plants and eukaryotic algae has been recognised for some time and contributes to photorespiration (Zelitch, 1971; Tolbert, 1974). The few prokaryotic phototrophs examined so far also produce glycollate during photosynthesis. For example, glycollate formation has been shown to occur by the blue-green algae *Anacystis nidulans* (Döhler and Braun, 1971), *Anabaena flos-aquae* and *Oscillatoria* (Cheng et al., 1972) and *Anabaena variabilis* and *Plectonema boryanum* (Han and Eley, 1973).

The photosynthetic bacteria *Chromatium D*, *Rhodospirillum rubrum* and *Rhodomicrobium vannielii* have also been found to form and excrete glycollate (Asami and Akazawa, 1974; Codd and Smith, 1974; Codd and Turnbull, 1975). The phenomenon therefore appears to be a feature of widely different types of phototrophic organisms.

Among the known routes of phototrophic glycollate formation is the oxygenolytic cleavage of RuDP to produce phosphoglycollate which is then hydrolyzed to glycollate (Zelitch, 1973, 1975). The oxygenation reaction is catalyzed by RuDP carboxylase (Bowes et al., 1971; Andrews et al., 1971). Since the RuDP carboxylase purified from the chemolithotroph *Alcaligenes eutrophus* has been found to exhibit oxygenase activity (Bowien et al., 1976), we have examined the ability of *A. eutrophus* to produce glycollate. The present paper reports on the formation, excretion and oxidation of glycollate by this non-photosynthetic autotroph and extends our preliminary communication on these findings (Codd et al., 1975).

MATERIALS AND METHODS

Organism and Cultivation. The PHB$^-$4 mutant of *Alcaligenes eutrophus H 16* was grown in batch culture either autotrophically as described previously (Bowien et al., 1976), or heterotrophically under air at 30° C with various organic carbon sources added to 0.5% (w/v) final concentration. Log-phase cells were used in all experiments.

Glycollate Excretion Experiments. Cells were harvested by centrifugation, washed and finally resuspended in 10 mM potassium

Abbreviations. DTE = dithioerythritol; EDTA = ethylenediamine tetraacetate; HPMS = 2-pyridyl-hydroxymethane sulphonic acid; RuDP = D-ribulose 1,5-diphosphate

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phosphate buffer (pH 8.0) containing 5 mM sodium bicarbonate, unless stated otherwise, at a density of 4 mg dry wt./ml. 50 ml of cell suspension were added to a 500 ml fluted Erlenmeyer flask equipped with a magnetic stirrer, side-arm and a head for gassing. The suspension was stirred at 750 rpm and the temperature maintained at 30° C. Gassing with $H_2/O_2/CO_2$ mixtures was performed at 400 ml/min, the mixtures being produced by means of calibrated Wösthoff pumps (H. Wösthoff oHG, D-4630 Bochum, W.-Germay). 2 ml samples of the suspension were removed periodically by syringe into ice-cold centrifuge tubes and quickly spun down in a bench centrifuge. The clear supernatant was decanted and 0.4 ml aliquots assayed for glycollate by spectrophotometric measurement of the 2,7-dihydroxynaphthalene derivative at 530 nm according to Calkins (1943).

Preparation of Cell-Free Extracts. All steps were performed at 0 – 4° C. Cells harvested by centrifugation were washed in the buffer appropriate to the assay, respun and broken by passage through a French pressure cell at 1500 kp/cm^2. The supernatant resulting from centrifugation at 45000 × g for 20 min was decanted and used for all enzyme assays.

Enzyme Assays. Phosphoglycollate Phosphatase. Cells were disrupted in 0.2 M cacodylate pH 7.0 and the enzyme activity in extracts assayed at 30° C by measuring liberated ortho-phosphate (Codd, 1970).

Glycollate Oxidation. Extracts were prepared in 0.1 M potassium phosphate buffer pH 8.0 and activity assayed by measuring glyoxylate formation as the absorbance of the phenylhydrazone derivative at 324 nm (Codd and Schmid, 1972).

RuDP Carboxylase. Cells were broken in 0.02 M Tris-HCl buffer (pH 7.8), containing 0.05 M $NaHCO_3$, 0.01 M $MgCl_2$, 0.001 M EDTA and 0.001 M DTE and enzyme activity determined by measuring RuDP-dependent $^{14}CO_2$ incorporation into acid-stable material as previously described (Bowien et al., 1976).

Protein was measured according to Lowry et al. (1951).

Chemicals. HPMS was purchased from the Aldrich Chemical Co., Milwaukee, U.S.A. Cacodylic acid, phosphoglycollate (trimonocyclo-hexylamine salt) and RuDP were obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A. Sodium ^{14}C-bicarbonate was purchased from the Radiochemical Centre, Amersham, England. Chloramphenicol came from Serva, Heidelberg, W.-Germany. Other reagents were bought from Merck, Darmstadt, W.-Germany, and used at the highest purity commercially available.

RESULTS AND DISCUSSION

Glycollate Production, Excretion and Oxidation

Autotrophically-grown cultures of *Alcaligenes eutrophus H 16*-PHB$^-$4 were first tested. With hydrogen alone, glycollate excretion rates were relatively low (Fig. 1). Excretion experiments were also performed in the presence of HPMS, an inhibitor of glycollate oxidation (Zelitch, 1971). The final HPMS concentration of 1 mM used caused only a 3.5% inhibition of carbon dioxide fixation during a 1 h-period (data not shown). Thus, glycollate excretion in the presence of HPMS may give an indirect indication of the rate of glycollate production. The low rate of glycollate excretion under 100% hydrogen (Fig. 1) is therefore taken to indicate a low rate of glycollate formation

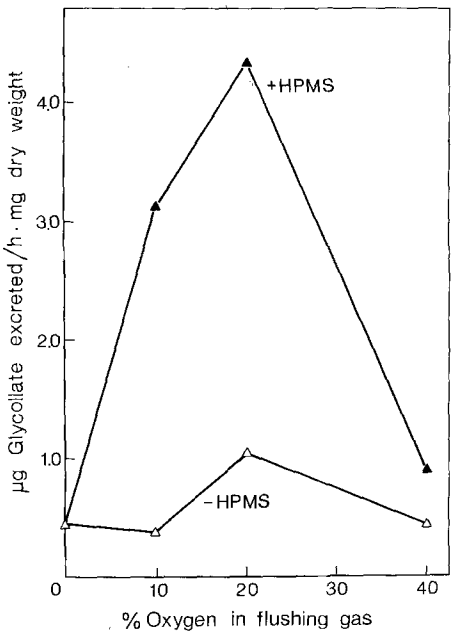

Fig. 1. Glycollate excretion by autotrophically grown *Alcaligenes eutrophus H 16*-PHB$^-$4. Log-phase cells were washed and resuspended in 10 mM potassium phosphate buffer (pH 8.0), containing 5 mM sodium bicarbonate, to a final density of 4 mg dry wt./ml and gassed with different percentages of oxygen in hydrogen. For experimental details see "Materials and Methods". ▲——▲ plus 1 mM HPMS; △——△ minus HPMS

under these conditions. The inclusion of oxygen in the gassing mixture caused marked increases in glycollate excretion (Fig. 1) particularly in the presence of HPMS. Experiments were run for 40 or 60 min and linear rates found. Glycollate excretion rates were maximal when cells were sparged with 20% oxygen in hydrogen. The inclusion of 40% oxygen caused a steep decline in excretion (Fig. 1). This effect may be due to the overall sensitivity of hydrogen bacteria to high oxygen partial pressures (Schlegel et al., 1961).

The effect upon glycollate excretion of including carbon dioxide in the flushing gas was also investigated. 10% carbon dioxide in hydrogen did not significantly affect the low rates of glycollate production and excretion previously found under hydrogen alone (Table 1). The addition of 10% carbon dioxide to the 20% oxygen in hydrogen, resulted in a marked decrease to almost the rates found under hydrogen alone (Table 1). Thus the stimulatory effect of oxygen upon glycollate production by *A. eutrophus* was essentially prevented by high carbon dioxide.

When heterotrophic cultures (grown on pyruvate) were examined, no glycollate was detected when cells were resuspended in either 10 mM phosphate buffer (pH 8.0), or in fresh pyruvate growth medium, when gassed with 20% oxygen in hydrogen (Table 1). Furthermore glycollate production by heterotrophic

Table 1. Effect of addition of high carbon dioxide to the gassing mixture upon glycollate excretion by autotrophic *Alcaligenes eutrophus H 16*-PHB$^-$4, and of heterotrophic growth on glycollate excretion[a]

Growth conditions	% Gas mixture supplied during excretion expts.			Glycollate excretion (μg/h mg dry weight)	
	O_2	CO_2	H_2	+HPMS	−HPMS
Autotrophic	0	0	100[b]	0.48	0.44
	0	10	90	0.55	0.38
	20	0	80[b]	4.31	1.05
	20	10	70	0.87	0.30
Heterotrophic (pyruvate)	20	0	80	ND[c]	ND

[a] Cells were used as given in the legend to Figure 1 and excretion experiments performed as in "Materials and Methods"
[b] Taken from Figure 1
[c] ND = not detectable when testing up to 0.4 ml of culture supernatant

Table 2. Activities of phosphoglycollate phosphatase, glycollate oxidoreductase and RuDP carboxylase in extracts from *A. eutrophus H 16*-PHB$^-$4 grown under different conditions

Culture conditions[a]	Phosphoglycollate phosphatase		Glycollate oxidoreductase		RuDP carboxylase	
	Sp. act.[b]	Rel. act.[c]	Sp. act.	Rel. act.	Sp. act.	Rel. act.
Autotrophic	12.1	100	8.0	100	263.3	100
Heterotrophic:						
Pyruvate	0.5	4	3.2	41	3.6	1
Fructose	2.7	22	3.3	42	49.3	19

[a] See "Materials and Methods" section
[b] Sp. act. = specific activity, expressed as nmoles substrate transformed/min · mg protein
[c] Rel. act. = relative activity, expressed as a percentage of the specific activity catalyzed by autotrophic extracts

cells in pyruvate medium under air was not found. Glycollate production therefore appeared to be only a property of autotrophic cells.

Glycollate production by phototrophs occurs via several routes including the production of an "active glycoaldehyde" transketolase complex, and the enzymic oxygenolysis of RuDP (Zeltich, 1971; Bassham and Kirk, 1973; Eichenbusch and Beck, 1973). The latter mechanism requires RuDP carboxylase to function as an oxygenase, producing equimolar amounts of phosphoglycerate and phosphoglycollate (Tolbert, 1974; McFadden and Tabita, 1974).

Glycollate formation via RuDP oxygenation also requires the participation of phosphoglycollate phosphatase. As shown in Table 2, phosphoglycollate phosphatase activity was readily detected in autotrophic extracts. Maximal glycollate production rates found (Fig. 1) approximate to 1.2 nmoles glycollate formed/min · mg protein, assuming that protein accounted for approximately 80% of dry weight (Bowien, unpublished results). The in vitro activity of phosphoglycollate phosphatase from autotrophic cultures (Table 2) was therefore considerably in excess of the observed rates of glycollate production.

The RuDP carboxylase purified from this organism does indeed show oxygenase activity, as detailed in our previous communication (Bowien et al., 1976). Furthermore, and approximate 1:1 stoichiometry has been found between O_2 consumed and phosphoglycerate formed as theoretically expected in the oxygenase reaction. Enzymic RuDP oxygenation may therefore contribute to the glycollate production and excretion observed with whole cells of *A. eutrophus*. The increased rates of glycollate excretion in the presence of HPMS (Fig. 1) suggested that some of the glycollate produced was being further metabolised. Indeed, extracts of autotrophic cells catalyzed the oxidation of glycollate to glyoxylate (Table 2).

Since glycollate production was found to occur by autotrophic cells, but not by cells cultured heterotrophically on pyruvate (Table 1), extracts from heterotrophic cultures were assayed for phosphoglycollate phosphatase and glycollate oxidoreductase. In both cases, levels of activity were considerably lower in

Table 3. Effect of transfer of *A. eutrophus H 16*-PHB$^-$4 from heterotrophic to autotrophic growth conditions on phosphoglycollate phosphatase, glycollate oxidoreductase and RuDP carboxylase activities

Culture conditions[a]	Phosphoglycollate phosphatase		Glycollate oxidoreductase		RuDP carboxylase	
	Sp. act.[b]	Rel. act.[c]	Sp. act.	Rel. act.	Sp. act.	Rel. act.
Heterotrophic (pyruvate)	0.5	1	3.2	1	9.0	1
Pyruvate cells transferred to autotrophic conditions[d]:						
plus chloramphenicol	0.7	1.3	3.5	1.1	4.0	0.4
minus chloramphenicol	12.5	25.0	8.2	2.58	71	7.9
Autotrophic control	12.2	24.4	8.0	2.53	70	7.8

[a] See "Materials and Methods" section
[b] Sp. act. = specific activity, expressed in nmoles substrate transformed/h · mg protein
[c] Rel. act. = relative activity, expressed in terms of the specific activity in extracts from pyruvate grown cells
[d] Log-phase cells washed and resuspended in autotrophic medium. Chloramphenicol added where indicated to 10^{-4} M

cells grown on pyruvate, and also when grown heterotrophically on fructose, than in extracts from autotrophic cultures (Table 2). These differences, although of varying magnitudes, generally resembled those found between RuDP carboxylase levels, in corresponding extracts (Table 2). The possible synthesis of phosphoglycollate phosphatase and the glycollate-oxidising enzyme during the establishment of autotrophic growth was investigated as follows. Log-phase cells grown on pyruvate were washed twice and transferred to autotrophic medium and growth conditions. After gassing for 7 h with a mixture of H_2, O_2 and CO_2 (80 : 10 : 10% by volume) cultures were harvested, disrupted and assayed. Parallel cultures were set up containing 10^{-4} M chloramphenicol. As shown in Table 3, phosphoglycollate phosphatase and glycollate oxidation rates increased during 7 h adaptation to autotrophy to about the levels previously measured with extracts from autotrophic cultures. The level of RuDP carboxylase also increased to that in autotrophic control cultures over this period. This was expected since it is well established that the synthesis of the RuDP carboxylase of *A. eutrophus H 16* is subject to control by induction and repression under similar conditions (Gottschalk et al., 1964; Eberhardt, 1966). The increases in activity of the three enzymes were prevented by chloramphenicol (Table 3), indicating their de novo synthesis during autotrophic adaption.

These observations are consistent with the finding that glycollate production was only found to occur by autotrophic, and not by heterotrophic cells (Table 1), and also with some earlier data reported by Hirsch et al. (1963). These workers detected the appearance of ^{14}C in glycollate during $^{14}CO_2$-labelling experiments using autotrophic *A. eutrohpus H 16* cultures, but not with cells grown heterotrohpically on succinate. The positive identification of the route(s) of glycollate production by *A. eutrophus* must await further investigation. However, the presence of RuDP oxygenase (Bowien et al., 1976), the stimulatory effect of oxygen at low partial volumes (Fig. 1) and the converse effect of high CO_2 on glycollate production (Table 1), and, finally, the presence of active phosphoglycollate phosphatase in autotrophic cell extracts (Table 2) are all consistent with autotrophic glycollate production by RuDP oxygenation. Labelling experiments using $^{18}O_2$ would permit some clarification of the contribution of this route in vivo. The data presented herein do not exclude the operation of alternate or concomitant pathways of glycollate formation. Indeed, as with *Rhodospirillum rubrum* (Anderson and Fuller, 1967; Codd and Smith, 1974), some glycollate formation by autotrophic *A. eutrophus* occurred under oxygen-free conditions (Fig. 1), which would preclude RuDP oxygenation.

It is of interest that our findings of autotrophic glycollate production by *A. eutrophus* (Codd et al., 1975) have recently been supported by similar observations with another chemolithotroph, *Thiobacillus neapolitanus* (Cohen and Kuenen, 1976). Future investigations may therefore determine whether glycollate production is a common feature of chemolithotrophic bacteria.

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