JOURNAL OF BACTERIOLOGY, July 1976, p. 516-522

Vol. 127, No. 1
Printed in U.S.A.

D-Ribulose-1,5-Bisphosphate Carboxylase and Polyhedral Inclusion Bodies in *Thiobacillus intermedius*

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Received for publication 20 February 1976

The growth-related parameters of *Thiobacillus intermedius*, cultured in glutamate-CO_2-$S_2O_3^{2-}$ medium, have been determined. After centrifugation at 48,000 × *g* for 1 h, 24% of the D-ribulose-1,5-bisphosphate carboxylase (RuBPCase) activity of the disrupted-cell suspensions obtained from CO_2-$S_2O_3^{2-}$- and glutamate-CO_2-$S_2O_3^{3-}$-grown cells could be sedimented, and the specific activities of this enzyme in the supernatant fractions were almost equivalent. The enzyme was stable in *T. intermedius* starved of thiosulfate in the presence and absence of glutamate, but a progressive decrease was evident in several growth cycles, each cycle supported by resupplementation of cells with thiosulfate. Polyhedral inclusion bodies were present in CO_2-$S_2O_3^{2-}$- and glutamate-CO_2-$S_2O_3^{2-}$-grown cells. The number of polyhedral bodies per cell increased during mixotrophic growth approximately in proportion to the observed increase in the specific activity of RuBPCase. RuBPCase could not be detected in *T. intermedius* grown heterotrophically on yeast extract, nor could polyhedral bodies be found.

Thiobacillus intermedius grows chemolithotrophically, utilizing any of several reduced inorganic sulfur compounds as a source of energy. Heterotrophic growth also occurs after a long lag in medium containing yeast extract alone or supplemented with glucose or glutamate. Optimal growth is observed in thiosulfate medium supplemented with an organic carbon source like glucose or glutamate (5, 6, 19). Accordingly, the organism has been termed as a mixotrophic chemolithotroph (15).

T. intermedius, mixotrophically grown in glutamate-thiosulfate medium, contains high levels of ribulose-1,5-bisphosphate carboxylase (RuBPCase). The observations of London and Rittenberg (6) suggest that a maximum of 70 to 80% of the carbon of such cells is derived from glutamate. Thus the high level of RuBPCase is ostensibly superfluous because CO_2 fixation by the reductive pentose phosphate pathway is presumably of less significance.

In recent years Shively and co-workers (2, 18) and other investigators (12) have reported the presence of polyhedral inclusion bodies in several thiobacilli including *T. intermedius* (18), which had been cultured on a minerals-thiosulfate medium supplemented with yeast extract. In a single electron micrograph, one or two polyhedral bodies were evident in one sectioned *T. intermedius* cell but were absent in a second cell, and assays for RuBPCase were not reported (18). Therefore, both the mode of growth and RuBPCase content of *T. intermedius* were uncertain.

More recently, the pioneering work of Shively et al. (17, 18) has resulted in the isolation of these polyhedral bodies from *T. neapolitanus* and, because they were rich in RuBPCase, has resulted in their designation as carboxysomes. However, *T. neapolitanus* is a strict autotroph and therefore can be grown only on CO_2. In contrast, nutritionally versatile *T. intermedius* can be cultured autotrophically, mixotrophically, and heterotrophically, and consequently there is the potential to manipulate RuBPCase levels. Thus, *T. intermedius* is an ideal organism with which to investigate the regulation of RuBPCase and the correlation between occurrence of polyhedral inclusion bodies and this enzyme. In the present report, we describe such an investigation.

MATERIALS AND METHODS

Bacterial culture and media. The *T. intermedius* culture was supplied by S. C. Rittenberg, University of California, Los Angeles. The culture was maintained at 30 C on autotrophic medium containing 2% agar and was subcultured at 7- to 10-day intervals. The autotrophic medium contained (grams per li-

ter): NH_4Cl, 1; $MgSO_4 \cdot 7H_2O$, 0.8; $CaCl_2 \cdot 2H_2O$, 0.2; KH_2PO_4, 0.4; $K_2PO_4 \cdot 3H_2O$, 0.6; ferric ammonium citrate (12.5% Fe), 0.04; and $Na_2S_2O_3$, 5.0. The medium was supplemented (per liter) with 10 ml of trace element solution (13), 1 mg of thiamine hydrochloride, and 0.1 mg of vitamin B12. Thiosulfate, phosphate, Ca^{2+}, vitamins, and organic supplements, when used, were each autoclaved separately and added to the rest of the sterile constituents after cooling. The final pH was 6.5. When used, neutralized L-glutamic acid was added to give a final concentration of 16 mM.

Growth conditions. Liquid cultures were incubated at 30 C on rotary shakers at 240 rpm, and growth was monitored turbidimetrically, using a Klett-Summerson photoelectric colorimeter equipped with a green filter. For inoculation, 1 volume of a preculture that had been grown at 30 C was transferred to 20 volumes of identical medium. Heterotrophic contaminants were routinely monitored by streaking on glucose-yeast extract (0.2% each) agar plates lacking thiosulfate. These could be easily distinguished by their colony morphology and rapid rate of growth on glucose-yeast extract plates without thiosulfate, a medium that supports extremely slow growth of *T. intermedius* (5). The relation between dry weight and Klett readings was established by using *T. intermedius* cells that had been washed with distilled water and dried at 105 C for 16 h.

Cell-free preparations. The cells were collected by centrifugation at 2 C, and the supernatant culture fluid was used as indicated for chemical determinations. The pelleted cells were suspended in TEMBD [made up with 20 mM tris(hydroxymethyl)aminomethane containing 1 mM ethylenediaminetetraacetic acid, 10 mM $MgCl_2 \cdot 6H_2O$, 50 mM $NaHCO_3$, and 1 mM dithiothreitol] buffer that had been adjusted to pH 8.0 at 25 C with HCl. After centrifugation, the wet pellet was stored at −20 C. For preparation of cell-free extracts, frozen cells were thawed, suspended usually in an equal volume of cold TEMBD buffer, and sonically disrupted for 30 s at 0 C at maximal power in a Brownwell Biosonik sonic disintegrator or passed through a prechilled French pressure cell at 16,000 to 20,000 lb/in^2. Disrupted cells were centrifuged at 10,000 × *g* for 45 min unless otherwise indicated. The resulting supernatant was used for RuBPCase assay. Protein was determined by the method of Lowry et al. (7), with bovine serum albumin as a standard and with an appropriate correction for tris(hydroxymethyl)-aminomethane or dithiothreitol.

RuBPCase assay. RuBPCase was assayed at 30 C as described by McFadden et al. (11) in a reaction mixture containing 0.4 mM dithiothreitol. One unit of enzyme catalyzes the RuBP-dependent fixation of 1 μmol of CO_2 per min. Specific activity is given in units per milligram of protein. The concentrations of Mg^{2+}, HCO_3^-, and ribulose bisphosphate used were saturating.

Chemical determinations. Thiosulfate was estimated iodometrically (3). Glutamic acid was determined with a Beckman 121 automatic amino acid analyzer, using norleucine as an internal standard. The probe for α-ketoacids was conducted by the method of Friedemann and Haugen (1).

Electron microscopy. Pellets of centrifuged cells were gently dislodged and fixed in 3% glutaraldehyde in 0.1 M potassium phosphate, pH 7.2. In some cases the fragments were embedded in 2% agar to avoid further disintegration. Specimens were rinsed with the same buffer and postfixed with 1% OsO_4, similarly buffered, for 1 h. All these steps were carried out at near 4 C. After dehydration in a graded ethanol series, the specimens were embedded in Epon and sectioned with a diamond knife on a Reichert OmU2 microtome. Electron micrographs were made at 75 kV in a Hitachi HU-11E microscope.

Thiosulfate resupplementation experiments. A 6-liter flask containing 2 liters of basal medium supplemented with 16 mM glutamate was inoculated with 100 ml of *T. intermedius* cells grown in medium that was identical and had depleted the thiosulfate. A 200-ml aliquot was immediately transferred to a sterile flask containing a Klett tube as side arm. The flasks were shaken at 240 rpm and incubated at 30 C. When the cells ceased to grow in the small flask, the pH, cell density, and thiosulfate level of the main flask were checked. The growth in the main flask was identical to that observed in the smaller flask. A 500-ml aliquot was removed from the main flask, and the cells were processed for RuBPCase assay. The culture remaining in the main flask was neutralized to pH 6.5 with sterile (10%) Na_2CO_3 and resupplemented with sterile (25%) $Na_2S_2O_3$ so that the final thiosulfate concentration was 0.5%. A portion (150 ml) of the neutralized and thiosulfate-resupplemented culture was transferred to another small flask with side arm, and the flasks were incubated as described earlier. The procedure of neutralization and thiosulfate resupplementation was repeated once more, during which the size of the main flask was reduced to 1 liter and the sample size for RuBPCase assay was correspondingly reduced. The remaining 150 ml of the culture after the third neutralization and thiosulfate resupplementation was incubated and processed for RuBPCase assay. The small flasks used for monitoring growth were incubated without neutralization and thiosulfate resupplementation. The cells were harvested at the time indicated and processed for RuBPCase assay.

Starvation experiments. Cells grown in glutamate-thiosulfate medium were harvested by centrifugation at 25 C, after depletion of thiosulfate. The cells were suspended in basal medium with and without glutamate (16 mM) in the absence of thiosulfate. In both cases cells were adjusted to 2.55 mg (dry weight)/ml, equivalent to 850 Klett units. Cell suspensions (70 ml) were shaken at 240 rpm in 500-ml flasks at 30 C under air. Ten-milliliter portions were removed periodically and chilled by transferring to glass centrifuge tubes in ice. Cells were collected by centrifugation at 2 C and washed once with cold TEMBD buffer. The collected cells were disrupted by passing through a prechilled French

pressure cell at 16,000 lb/in². The resultant suspension was centrifuged at 48,000 × *g* for 1 h, and the supernatant fluid was used for the RuBPCase assay.

RESULTS

Growth characteristics. The growth of *T. intermedius* in thiosulfate-glutamate (16 mM) began with almost simultaneous oxidation of thiosulfate, as evident from the decrease in pH and thiosulfate concentration (Fig. 1). The pH of the medium continued to decrease, albeit slowly, after depletion of thiosulfate, and the final pH observed was 2.1.

The net rate of appearance of RuBPCase as measured from the time course of increase in specific activity slightly exceeded cell growth during the exponential phase. A slight decrease in specific activity was observed during the stationary phase.

A complete analysis of the culture supernatant showed utilization of glutamic acid (Fig. 1). No other amino acid or 2,4-dinitrophenyl hydrazine-reacting material could be detected in the culture filtrates.

RuBPCase after resupplementation of thiosulfate. Early in the studies, the inability of cells to grow in 1% thiosulfate was recognized; this was circumvented by lowering the thiosulfate concentration to 0.5%. At this concentration growth was limited by thiosulfate, and a test was conducted to assess the effect of providing fresh thiosulfate. First the pH of the thiosulfate-depleted culture was adjusted to 6.5 with sterile Na_2CO_3, and fresh sterile thiosulfate was then added to a final concentration of 0.5%. Subsequent exponential growth resumed without any appreciable lag. The procedure of neutralization and resupplementation with thiosulfate could be continued in a 100-ml culture for three cycles, i.e., until 1.5 g of thiosulfate had been consumed, but could not be continued beyond that point. Each thiosulfate resupplementation resulted in an almost equal increase in cell density (Fig. 2). In Fig. 2 a linear decrease in RuBPCase specific activity is revealed just before each neutralization and resupplementation. The total decrease in specific activity amounted to 60% and occasionally was even greater. Further incubation for 22 h of neutralized cells that did not grow any more in the presence of added thiosulfate also showed a decrease in specific activity equivalent to the two previous cycles. In contrast, cells that had been incubated in the thiosulfate-depleted culture medium after the first and second growth

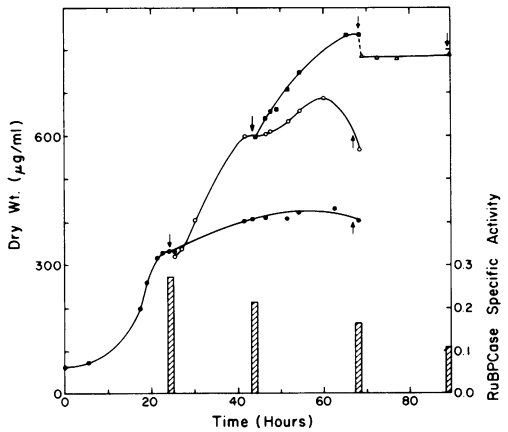

FIG. 2. *Growth of T. intermedius with resupplementation of thiosulfate. The downward arrows identify the time of neutralization and supplementation with thiosulfate and of sampling. The continuation of the curves bearing the same symbols denotes unsupplemented cultures. The pH was 2.9 to 3.0 at the points specified by upward vertical arrows. Bar graphs show RuBPCase specific activity.*

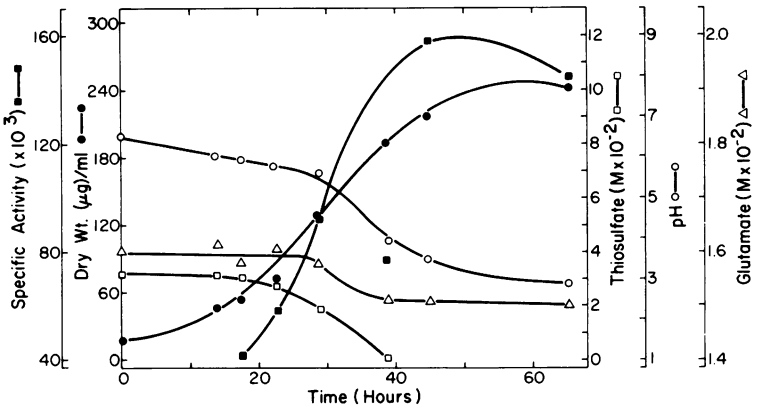

FIG. 1. *Growth-related parameters of T. intermedius at 30 C, shaken at 240 rpm.*

cycles and harvested at the times specified by upward vertical arrows showed no decrease in specific activity for RuBPCase. In fact, in such suspensions 32 and 13% increases in specific activity were observed, respectively. During incubation of thiosulfate-depleted cells, slight growth reminiscent of diauxie was apparent and could possibly have been due to energy yielded by the various intracellular intermediates (20) of thiosulfate oxidation.

Effect of starvation for thiosulfate in the presence and absence of glutamate. With *Hydrogenomonas eutropha* that had depleted fructose, a pronounced rapid decrease in RuBPCase occurred upon entry into the stationary phase. This decay appeared to be an energy-dependent process (16) and could be obtained with cells harvested in the log phase and subsequently starved for fructose. The presence of fructose prevented the decay. Thiosulfate-depleted cultures of *T. intermedius* on prolonged incubation in the presence of glutamate did not show any decrease in RuBPCase specific activity, whereas the enzyme activity decreased during successive growth cycles in the presence of glutamate and thiosulfate (Fig. 2). In an effort to clarify the cause of the decrease of RuBPCase specific activity, *T. intermedius* cells harvested from log-phase cultures were incubated in the absence of thiosulfate, the source of energy, with and without 16 mM glutamate in basal mineral salt medium. The conditions were similar to those for a freshly inoculated culture. No significant decrease in specific activity was observed during incubation for 4 h at 30 C (Table 1).

Distribution of protein and RuBPCase activity. RuBPCase activity could not be detected in cells grown in yeast extract-thiosulfate medium (Table 2). About 73% of the enzyme was found in the 48,000 × *g* (1 h) supernatant, and the rest was found in the pellet after autotrophic or mixotrophic growth. The specific activities of the high-speed supernatants derived from autotrophically and mixotrophically grown cells were almost equivalent. These results are in basic agreement with those of London and Rittenberg (6), although these workers observed a significantly lowered RuBPCase level after mixotrophic growth on glutamate.

Electron microscopy. Electron microscopic examination of thin sections of autotrophically grown *T. intermedius* showed four to six polyhedral bodies per cell in numerous fields. These bodies were located in the nuclear region along the central axis of the cell. The largest average diameter of these bodies was about 100 nm in both longitudinal and transverse sections (Fig. 3). *T. intermedius* grown in yeast extract-thiosulfate medium did not show any polyhedral bodies, but these cells had diffuse intracytoplasmic electron-dense material.

Glutamate-thiosulfate-grown *T. intermedius* cells, like those grown autotrophically, contained polyhedral bodies (Fig. 3). In the experiments described in Fig. 1, these bodies were found to be present during all phases of growth. However, the exponential-phase cells (18 h) contained one to four polyhedral bodies per cell, whereas the same culture after 44 h contained five to twelve polyhedral bodies (Fig. 4) per cell; thus the number of polyhedral inclusion bodies showed a rough correlation with the specific activity of RuBPCase. Indeed, both the number of polyhedral bodies per cell and the specific activity of RuBPCase peaked almost simultaneously.

The isolation of polyhedral bodies from *T.*

TABLE 1. *Effect of carbon starvation upon RuBPCase in the absence of thiosulfate*

Period of starvation (min)	Sp act			
	16 mM Glutamate		No glutamate	
	Disrupted-cell suspension[a]	Supernatant[b]	Disrupted-cell suspension[a]	Supernatant[b]
30	0.058	0.139	0.059	0.130
60	0.062	0.129	0.052	0.158
135	0.054	0.159	0.057	0.126
250	0.056	0.138	0.066	0.161

[a] Disrupted in French pressure cell at 16,000 to 20,000 lb/in².

[b] Supernatant derived from centrifugation of the disrupted cell suspension at 48,000 × *g* for 1 h.

TABLE 2. *Distribution of protein and RuBPCase in T. intermedius*

Medium	Disrupted-cell fraction	Protein (%)	Activity (%)	Sp act
CO_2-$S_2O_3^{2-}$	Suspension[a]	(100)	(100)	0.18
	Supernatant[b]	42	73	0.38
	Pellet[c]	46	24	0.12
Glutamate-CO_2-$S_2O_3^{2-}$	Suspension	(100)	(100)	0.11
	Supernatant	20	72	0.41
	Pellet	74	24	0.04
Yeast extract[d]-CO_2-$S_2O_3^{2-}$		ND[e]	ND	ND

[a] Disrupted in a prechilled French pressure cell at 16,000 to 20,000 lb/in².

[b] 48,000 × *g*, 1 h.

[c] 48,000 × *g*, 1 h.

[d] 0.1%.

[e] ND, Not detectable.

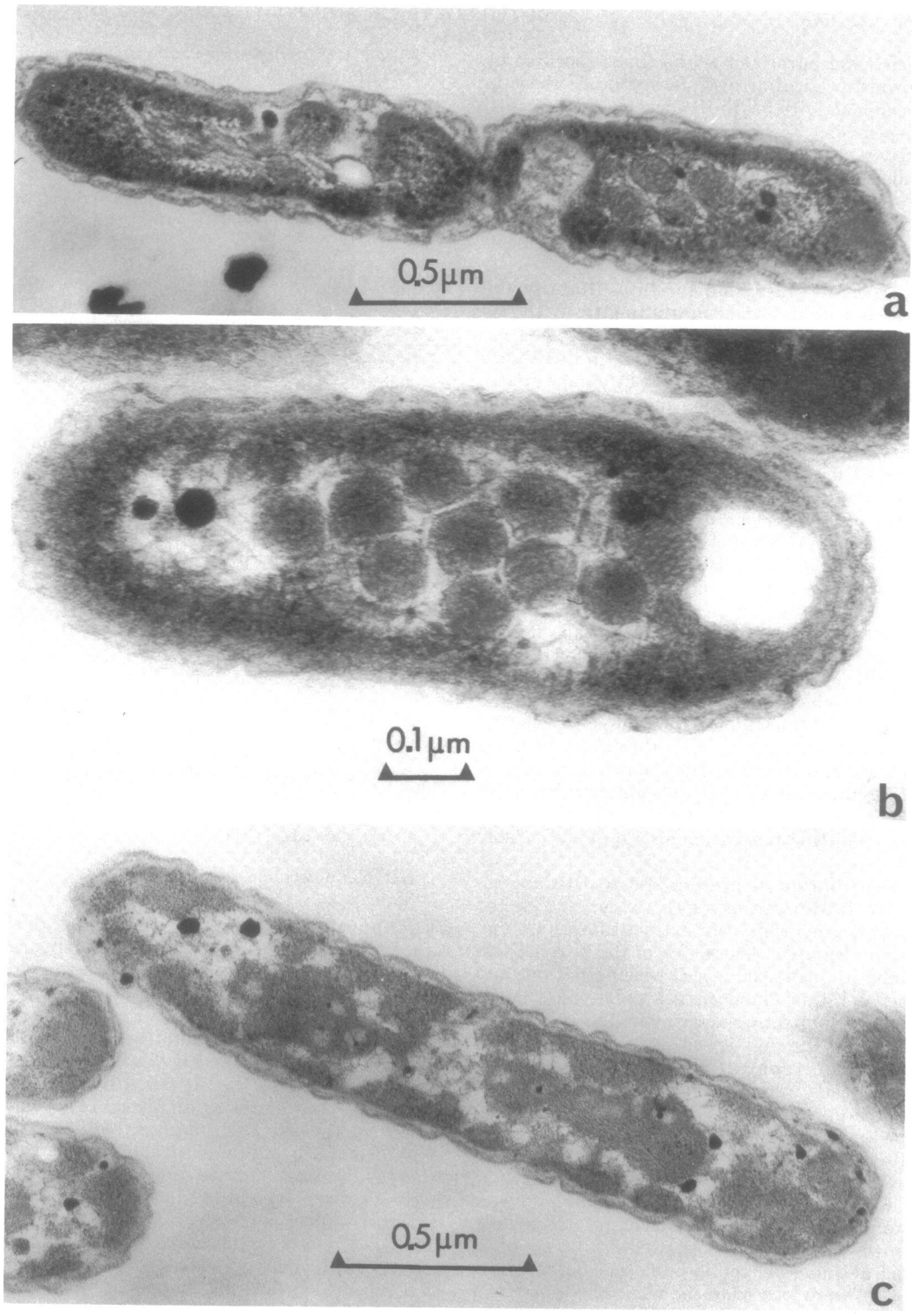

FIG. 3. *Electron photomicrographs of thin sections of representative cells harvested in the log phase after growth in (a) CO_2-$S_2O_3^{2-}$, (b) glutamate-CO_2-$S_2O_3^{2-}$, and (c) yeast extract-CO_2-$S_2O_3^{2-}$ media. The cell shown in (b) was from a culture grown in glutamate-CO_2-$S_2O_3^{2-}$ medium just before the second thiosulfate resupplementation (cf. Fig. 2).*

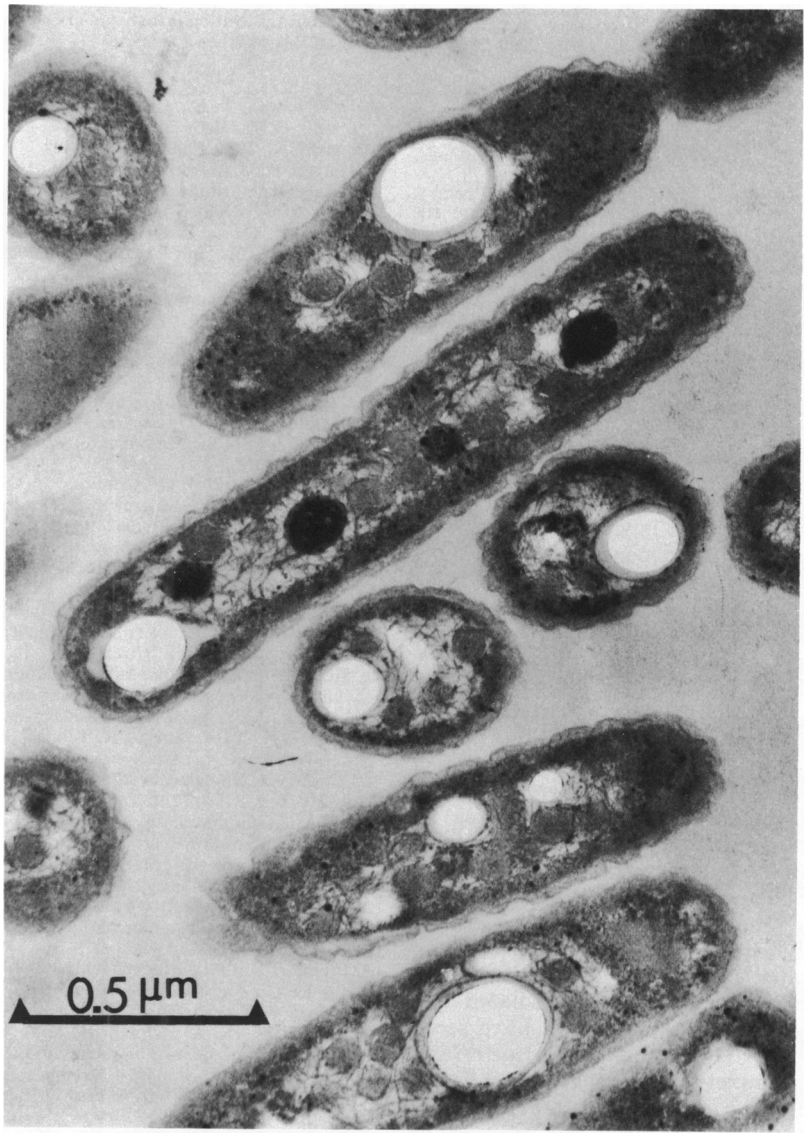

FIG. 4. *Electron photomicrograph of thin sections of representative cells harvested after 44 h in glutamate-CO_2-$S_2O_3^{2-}$ medium (cf. Fig. 1).*

neapolitanus on a sucrose density gradient has been reported (16), but has not proven successful from either frozen or freshly harvested cells of *T. intermedius*. However, in the 48,000 × *g* pellet derived from glutamate-grown cells, a large number of polyhedral bodies were visible by electron microscopy. It seems that the *T. intermedius* polyhedral bodies are either more fragile and/or denser than those of *T. neapolitanus*.

DISCUSSION

Growth rates and yields of *T. intermedius* when cultured on CO_2-glutamate are much higher than those realized by growth under strictly autotrophic growth conditions. Therefore, experimental strategies may be developed that lead to high yields of RuBPCase-enriched cells in relatively short times. Because this enzyme is of evolutionary interest (8, 10), such an

approach is significant and, in fact, has facilitated the isolation and characterization of RuBPCase described in the companion paper (14).

RuBPCase is much more stable in *T. intermedius* than in *H. eutropha*. For example, removal of the organic carbon growth substrate, glutamate, has no effect upon this enzyme in cells incubated for up to 4 h, whereas removal of the growth substrate, fructose, from *H. eutropha* triggers a rapid decay with a half-time of 15 min at 30 C. The enzyme is also quite stable in stationary-phase cultures of *T. intermedius* that are limited in growth by thiosulfate. Stationary-phase cultures of *T. denitrificans* are similar in this regard (8). In contrast, cells of *H. eutropha* entering the stationary phase because of the depletion of fructose rapidly lose RuBPCase (4). It is evident that the regulation of RuBPCase is variable among autotrophic bacteria and depends critically upon the growth conditions.

The regulation of RuBPCase in *T. intermedius* is of special interest because of the finding of polyhedral inclusion bodies in several of the thiobacilli (2, 12, 18) and the finding that these bodies in *T. neapolitanus* are enriched in this enzyme (16, 17). Our data establishing the simultaneous absence of the bodies and RuBPCase in yeast extract-grown *T. intermedius* and the presence of these bodies in autotrophically grown RuBPCase-enriched cells suggests but does not prove a functional role of these polyhedral inclusion bodies in CO_2 assimilation. Moreover, the parallel increase between polyhedral bodies and RuBPCase in mixotrophically grown cells suggests such a role. Although w have not yet isolated the bodies, in several experiments we have sedimented 25 to 40% of the total RuBPCase activity from mixotrophically grown cells at 48,000 $\times$ *g* (for 1 h) and have established that this pellet is rich in polyhedral bodies. In light of these findings, it will be of considerable interest to determine whether RuBPCase and other enzymes of the Calvin cycle are indeed sequestered in these bodies and to ascertain their function. *T. intermedius* is an ideal organism for these studies because of its facultative nature.

ACKNOWLEDGMENTS

We thank S. Gurusiddaiah, Manager of the Bioanalytical Laboratory, Washington State University, for conducting the amino acid analyses of culture filtrates and the glutamate measurements. We also acknowledge the support by the Public Health Service through a research grant (GM-19972, from the National Institute of General Medical Sciences) to B.A.M.

LITERATURE CITED

1. **Friedemann, T. E., and G. E. Haugen.** 1943. Pyruvic acid. II. The determination of keto-acids in blood and urine. J. Biol. Chem. 147:415–422.
2. **Holt, S. C., J. M. Shively, and J. W. Greenawalt.** 1974. Fine structure of selected species of the genus *Thiobacillus* as revealed by chemical fixation and freeze-etching. Can. J. Microbiol. 20:1347–1351.
3. **Kolthoff, I. M., and E. B. Sandell.** 1952. Text book of quantitative inorganic analysis, 3rd ed. MacMillan Publishing Co., New York.
4. **Kuehn, G. D., and B. A. McFadden.** 1968. Factors affecting the synthesis and degradation of ribulose-1,5-diphosphate carboxylase in *Hydrogenomonas facilis* and *Hydrogenomonas eutropha*. J. Bacteriol. 95:937–946.
5. **London, J.** 1963. *Thiobacillus intermedius* nov. sp. A novel type of facultative autotroph. Arch. Mikrobiol. 46:329–337.
6. **London, J., and S. C. Rittenberg.** 1966. Effects of organic matter on the growth of *Thiobacillus intermedius*. J. Bacteriol. 91:1062–1069.
7. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
8. **McFadden, B. A.** 1973. Autotrophic CO_2 assimilation and the evolution of ribulose diphosphate carboxylase. Bacteriol. Rev. 37:289–319.
9. **McFadden, B. A., and A. R. Denend.** 1972. Ribulose diphosphate carboxylase from autotrophic microorganisms. J. Bacteriol. 110:633–642.
10. **McFadden, B. A., and F. R. Tabita.** 1974. D-ribulose-1,5-diphosphate carboxylase and the evolution of autotrophy. BioSystems 6:93–112.
11. **McFadden, B. A., F. R. Tabita, and G. D. Kuehn.** 1975. Ribulose diphosphate carboxylase from the hydrogen bacteria and *Rhodospirillum rubrum*, p. 461–472. *In* W. A. Wood (ed.), Methods in enzymology, vol. 42. Academic Press Inc., New York.
12. **Murphy, J. R., A. R. Girard, and R. C. Tilton.** 1974. Ultrastructure of a marine *Thiobacillus*. Can. J. Microbiol. 85:130–138.
13. **Pfennig, N.** 1961. Eine vollsynthesche Nährlösung zur selectien Amreicherung einiger Schweffelpurpurbacterien. Naturwissenschaften 48:136.
14. **Purohit, K., B. A. McFadden, and A. L. Cohen.** 1976. Purification, quaternary structure, composition, and properties of D-ribulose-1,5-bisphosphate carboxylase from *Thiobacillus intermedius*. J. Bacteriol. 127:505–515.
15. **Rittenberg, S. C.** 1969. The roles of exogenous organic matter in the physiology of chemolithotrophic bacteria. Adv. Microb. Physiol. 3:159–196.
16. **Shively, J. M., F. C. Ball, D. H. Brown, and R. E. Sanders.** 1973. Functional organelles in procaryotes: polyhedral inclusions (carboxysomes) in *Thiobacillus neapolitanus*. Science 182:584–586.
17. **Shively, J. M., F. C. Ball, and B. W. Kline.** 1973. Electron microscopy of the carboxysomes (polyhedral bodies) of *Thiobacillus neapolitanus*. J. Bacteriol. 116:1405–1411.
18. **Shively, J. M., G. L. Decker, and J. W. Greenawalt.** 1970. Comparative ultrastructure of *Thiobacilli*. J. Bacteriol. 101:618–627.
19. **Smith, D. W., and S. C. Rittenberg.** 1974. On the sulfur-source requirement for growth of *Thiobacillus intermedius*. Arch. Microbiol. 100:65–71.
20. **Suzuki, I.** 1974. Mechanism of inorganic oxidation and energy coupling. Annu. Rev. Microbiol. 28:85–101.