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Carbon Dioxide Assimilation by *Thiobacillus novellus* Under Nutrient-Limited Mixotrophic Conditions

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The contribution of CO_2 to cell material synthesis in *Thiobacillus novellus* under nutrient-limited conditions was estimated by comparing $^{14}CO_2$ uptake rates of steady-state autotrophic cultures with that of heterotrophic and mixotrophic cultures at a given dilution rate. Under heterotrophic conditions, some 13% of the cell carbon was derived from CO_2; this is similar to the usual anaplerotic CO_2 fixation in batch cultures of heterotrophic bacteria. Under mixotrophic conditions, the contribution of CO_2 to cell material synthesis increased with increasing $S_2O_3^{2-}$-to-glucose ratio in the medium inflow; at a ratio of 10, ca. 32% of the cell carbon was synthesized from CO_2. We speculate that the use of CO_2 as carbon source, even when the glucose provided is sufficient to fulfill the biosynthetic needs, may augment the growth rate of the bacterium under such nutrient-limited conditions and could therefore be of survival value in nature. Some of the CO_2 assimilated was excreted into the medium as organic compounds under all growth conditions, but in large amounts only in autotrophic environments as very low dilution rates.

Facultative chemolithotrophs such as *Thiobacillus novellus* have the capacity for autotrophic as well as heterotrophic growth. Since in nature these bacteria are likely to encounter autotrophic and heterotrophic substrates simultaneously, their mixotrophic potential, i.e., the capacity to concurrently utilize organic and inorganic growth substrates, is of considerable interest (9).

Previous studies from this laboratory (5, 13, 15) have shown that the response of *T. novellus* to mixotrophic conditions depends on the nutritional status of the environment. Under nutrient-excess conditions, where energy overproduction is an evident threat, two strategies are used to circumvent this problem: repression and inhibition of metabolic enzymes, which reduce the rate of organic (glucose) and inorganic (thiosulfate) substrate utilization, and partial uncoupling of substrate oxidation from energy generation. This results in a lack of enhanced growth rate and yield in nutrient-excess mixotrophic environment, even though both glucose and thiosulfate are concurrently and completely utilized. In contrast, under nutrient limitation, lack of enzyme repression and the uncoupling phenomenon ensure maximal and efficient utilization of both substrates with the result that biomass formation in such mixotrophic cultures is nearly additive of that of heterotrophic and autotrophic cultures.

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We also showed that under nutrient-limited mixotrophic conditions both thiosulfate and glucose contributed to energy generation and suggested that CO_2 served as a carbon source along with glucose under these conditions (5). Since the publication of our work (5, 13, 15), two papers dealing with another facultative chemolithotroph, *Thiobacillus* A2, have appeared (3, 18) which strongly suggest a role for CO_2 during nutrient-limited mixotrophic growth. This role was inferred either from the presence of the CO_2-fixing potential in mixotrophically grown cells (3, 18) or from the extent of labeled acetate assimilation during mixotrophic growth (3). In this paper, we deal with the assimilation of $^{14}CO_2$ itself by steady-state cells of *T. novellus* in various nutrient-limited growth environments. We realized that because of the "open" nature of the steady-state growth conditions, it would be difficult to obtain a direct quantitative estimate of the cell carbon derived from CO_2: such an estimate would necessitate maintenance of $^{14}CO_2$ in the chemostat system at a precisely known specific activity for substantial time periods, which presents significant technical problems. However, since the extent of CO_2 contribution to cell material synthesis under strict autotrophic conditions has to be very nearly 100%, we reasoned that determination of relative initial rates of $^{14}CO_2$ uptake by steady-state cells in different environments at similar values of D, aeration, total CO_2, etc., should provide a method for assessing the contribution of CO_2 to

cell material synthesis under various conditions. Accordingly, *T. novellus* was grown to a steady state under different nutrient-limited conditions in a chemostat, the cultures were pulsed with $NaH^{14}CO_3$, and the initial rates of $^{14}CO_2$ uptake by the cells were determined. While this investigation was in progress, Cohen et al. (2) reported that *T. neapolitanus* excreted significant amounts of the assimilated CO_2 into the medium, and we decided to determine whether this was also true of *T. novellus* grown in various environments.

MATERIALS AND METHODS

Organism and growth procedure. We used the ATCC type strain of *T. novellus* (no. 8073) used in our previous studies (5, 12, 13, 15). Composition of the basal medium, maintenance of stock cultures, the design of the chemostat (4), and chemostat cultivation were exactly as previously described (4, 5, 13, 15); the working volume of the culture vessel was 500 ml. The concentration of the growth substrates in the different inflow media used is specified where appropriate. These concentrations were within the range of those at which, according to previous studies, complete utilization of the carbon and energy substrates occurs in steady-state cultures. All media except the one supplemented with biotin (see below) contained 0.03% yeast extract to satisfy the biotin requirement of *T. novellus* (11).

Determination of $^{14}CO_2$ assimilation by the cells and excretion of products of $^{14}CO_2$ assimilation. *T. novellus* cultures in various environments were allowed to reach a steady state (i.e., they were allowed to grow for at least 5 volume changes under a given set of conditions) and were then pulsed with a filter-sterilized solution (10 ml) of 380 μmol of $NaH^{14}CO_3$ (Amersham Corp., Arlington Heights, Ill.) containing 400 μCi of radioactivity. Since CO_2 is rapidly lost to air from a stirred solution of $NaHCO_3$, the chemostat air supply was made recyclable at the time of $NaH^{14}CO_3$ addition to the culture vessel. This was accomplished by placing the air pump between the product reservoir and the culture vessel, so that the effluent air was pumped back into the growth vessel. Other conditions, e.g., medium flow rate, stirring, etc., remained unaltered, so that the steady-state growth conditions were not disturbed. Under these conditions the uptake of $^{14}CO_2$ by cells remained linear with time for 1 to 2 h (see Fig. 1). Calculations showed that CO_2 and O_2 remained at saturating levels in the recycled air during this period.

Samples of 7 ml were removed from the culture immediately after the addition of $NaH^{14}CO_3$ and at 15- to 30-min intervals thereafter. These samples were treated and counted to obtain the following information. (i) For total $^{14}CO_2$ counts in culture, 100 μl of the culture was counted. (ii) For total $^{14}CO_2$ assimilation, 1 ml of the sample was acidified to a pH of approximately 3.5 by adding 10 μl of 5 M HCl and gently aerating for 5 min to eliminate dissolved $^{14}CO_2$. The efficacy of the procedure in removing CO_2 was checked in separate control experiments. For this measurement, 100 μl of the treated sample was counted. (iii) For products of $^{14}CO_2$ assimilation that were excreted by the cells, 5 ml of the sample was filtered (using a 0.45-μm membrane filter), and the filtrate was rendered free of CO_2 as described above. For this, 100 μl of the treated filtrate was counted. (iv) $^{14}CO_2$ assimilated and retained by the cells was estimated by subtracting (iii) from (ii); in some experiments direct counting of washed cells on filters was also used, and the results of the two procedures were found to agree closely.

All counts were made in duplicate, and average values are presented. The counting cocktail used consisted of a 4:1 mixture of toluene and 2-methoxyethanol and 6.4 g of 2,5-diphenyloxazole per liter.

Control experiments showed that the measured rate of $^{14}CO_2$ uptake under a given set of conditions decreased exponentially with increasing steady-state biomass of the culture. Therefore, in choosing the concentration of the limiting nutrient(s) in the various inflow media, an attempt was made to obtain similar steady-state biomass values under the different growth conditions. Variations in steady-state biomass did occur, however, under different growth conditions; these were generally small and were corrected for by the use of a standard curve that related $^{14}CO_2$ uptake rate with steady-state cell biomass under otherwise identical conditions.

RESULTS

Contribution of CO_2 to cell biomass formation in different environments. Figure 1 shows the typical kinetics of $^{14}CO_2$ uptake by steady-state cells in different environments. The uptake rates remained linear from 0.75 to 1.5 h, and it is evident that, at a fixed D value, the rate was highest under autotrophic conditions, lowest under heterotrophic conditions, and intermediate under mixotrophic conditions.

To calculate the contribution of CO_2 to the synthesis of cell material under different conditions from such slopes, it was necessary to determine whether CO_2 was the sole source of carbon under autotrophic conditions. Like all of the media used in this and previous studies (5, 13, 15), the autotrophic medium contained a small amount (0.03%) of yeast extract to satisfy the biotin requirement (11) of *T. novellus*, and it was conceivable that this substrate supplied significant amounts of cell carbon for biosynthesis. To check this point, kinetics of $^{14}CO_2$ uptake were determined for thiosulfate-yeast extract (1 and 0.03%, respectively) and thiosulfate-biotin (1% and 10^{-5} M, respectively) cultures grown at a D value of 0.02 h^{-1}. The former assimilated CO_2 at a rate some 90% that of the latter. The amount of biotin supplied in the biotin-containing medium was too low to have contributed a significant amount of carbon for cell biosynthesis, and it is therefore reasonable to assume that in this medium essentially 100% of the cell carbon was derived from CO_2. It follows that in thiosulfate-yeast extract medium, some 90% of the cell carbon came from CO_2 and the rest from

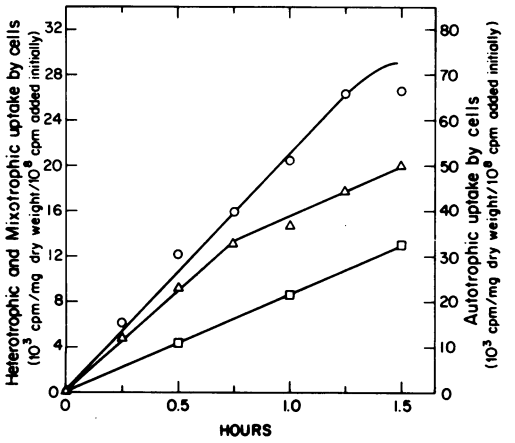

FIG. 1. $^{14}CO_2$ uptake by *T. novellus* cells during steady-state growth at $D = 0.05\ h^{-1}$ under various conditions. The concentration of the limiting substrates in the inflow medium were: 40.29 mM $S_2O_3^{2-}$ (autotrophic culture, ○); 4.0 mM glucose (heterotrophic culture, □); or 15.17 mM $S_2O_3^{2-}$ plus 1.67 mM glucose (mixotrophic culture, △). Note the difference in scales of the autotrophic uptake rates and that of the other cultures.

the precursors supplied by yeast extract. Thus, in calculating the contribution of CO_2 to cell material synthesis under heterotrophic and mixotrophic conditions, the $^{14}CO_2$ assimilation slope of the yeast extract containing autotrophic cultures was taken to represent provision of 90% of cell carbon from CO_2. It would, of course, have been simpler to use biotin rather than yeast extract in all the experiments. This was not done for two reasons: the μ_{max} in thiosulfate-biotin medium is around $0.025\ h^{-1}$, so that the higher D value could not have been studied with such a medium; also, yeast extract was used in previous studies (5, 13, 15), and we wished to keep the growth conditions comparable.

Table 1 presents the rates of $^{14}CO_2$ assimilation by cells and the contribution of CO_2 to cell material synthesis under different steady-state growth conditions. Under heterotrophic conditions, some 13% of the cell carbon was derived from CO_2. This value is comparable to the 10% amount that is generally derived from CO_2 by anaplerotic CO_2 fixation during heterotrophic growth in batch culture. The contribution of CO_2 to the synthesis of cell material under mixotrophic conditions depended on the ratio of $S_2O_3^{2-}$ to glucose in the inflow medium. When this ratio was 5, ca. 17% of cell carbon came from CO_2, i.e., somewhat higher than under heterotrophic conditions. Reducing this ratio to 2.5 decreased CO_2 assimilation to the range of the heterotrophic culture, but when the ratio was increased to 10, some 32% of the cell carbon was derived from CO_2. Under the latter conditions the steady-state biomass was 23 mg of cell dry wt, i.e., ca. 11.5 mg of cell carbon/100 ml of culture, and the amount of glucose supplied in the inflow medium (1.67 mM) was equivalent to 12 mg of carbon/100 ml of medium. Thus, the available glucose was just enough to serve as sole carbon source under these conditions. The fact that 32% of the cellular carbon was derived from CO_2 indicates that an equivalent amount of the available glucose was respired. There must have been a net loss of energy in these conversions, since the amount of energy gained from glucose oxidation is likely to be considerably less than that expended in generating an equivalent amount of organic material from CO_2.

Excretion of products of $^{14}CO_2$ assimilation. In heterotrophic cultures, the products of CO_2 assimilation excreted into the medium as organic material were small, some 3 to 4% of the total CO_2 fixed, or 0.3 to 0.6% of the total biomass synthesized (Table 2). The mixotrophic cultures showed slightly higher amounts, ranging from 4 to 8% of the total CO_2 assimilated, but still no more than 1.1% of the total biomass formed. In contrast, the autotrophic cultures excreted up to 14% of the total CO_2 fixed, i.e., ca. 16% of the biomass synthesized at $D = 0.02\ h^{-1}$; this excretion decreased sharply at higher D values, but still remained significantly greater than in other media. Curiously, when biotin replaced yeast extract in autotrophic medium, the excretion of ^{14}C-containing organic compounds doubled, corresponding to ca. 40% of the biomass synthesized under these conditions.

DISCUSSION

As we had expected, measurement of short-term uptake rates of CO_2 by steady-state cells of *T. novellus* pulsed with $H^{14}CO_3^-$ made it feasible to assess the contribution of CO_2 to cell material synthesis under various nutrient-limited conditions. Such rates could not provide a reliable direct quantitative measure of the extent of CO_2 contribution to cell synthesis mainly because the amount of CO_2 in the chemostat system and hence its specific activity changed during the experiment and were not exactly known. Nevertheless, the same qualitative picture of the relative contribution of CO_2 to cell matrial synthesis under various conditions as that assessed by the indirect method used here emerged when the data were processed to yield quantitatively the amount of CO_2 fixed under various conditions. Similarly, allowing for the uncertainties surrounding the assumptions on which the direct quantitative calculations must be based, their results were in reasonable agreement with the amount of carbon assimilation predicted by the indirect method used here. For

TABLE 1. Rate of $^{14}CO_2$ assimilation and percentage of carbon derived from CO_2 under various steady-state growth conditions

Growth environment	$S_2O_3^{2-}$/glucose ratio	Dilution rate (h^{-1})	Steady-state biomass (mg of cell dry wt/100 ml of culture)	Rate of $^{14}CO_2$ assimilation[a]	Cell carbon derived from CO_2 (% of total)[b]
Autotrophic					
40.29 mM $S_2O_3^{2-}$		0.02	18	54,000	90[c]
40.29 mM $S_2O_3^{2-}$		0.05	25	52,900	90[c]
Heterotrophic					
7.28 mM glucose		0.02	52	7,700	13
4.00 mM glucose		0.05	37	7,600	13
Mixotrophic					
3.89 mM glucose + 18.82 mM $S_2O_3^{2-}$	5	0.02	26	9,900	17
3.33 mM glucose + 15.17 mM $S_2O_3^{2-}$	5	0.05	32	9,100	16
3.33 mM glucose + 7.59 mM $S_2O_3^{2-}$	2.5	0.05	25	6,100	10
1.67 mM glucose + 15.17 mM $S_2O_3^{2-}$	10	0.05	23	18,500	32

[a] Counts per minute of $^{14}CO_2$ taken up by the cells per milligram of cell dry wt per hour per 10^8 cpm added initially.

[b] Calculated from the autotrophic rate, at corresponding D values, on the assumption that 90% of the cell carbon was derived from CO_2 under autotrophic conditions.

[c] See text.

instance, at $D = 0.05\ h^{-1}$, the cell material synthesized was 50 μg of dry weight h^{-1}, which is roughly equivalent to the assimilation of 25 μg of carbon h^{-1}. The indirect method used in this study has shown that in yeast-extract-containing autotrophic media, ca. 90% of the carbon comes from CO_2, and therefore we should find $^{14}CO_2$ fixation equivalent to the assimilation of 22.5 μg of carbon h^{-1}. The counts from $^{14}CO_2$ actually assimilated in this culture (52,900 cpm/mg of biomass per h per 10^8 cpm initially added; Table 1) very roughly correspond to 9.3 μg of carbon assimilated $h.^{-1}$ (This value is based on the assumption that at 1 h after the addition of $H^{14}CO_3^-$ pulse, the chemostat system contained approximately 1,460 μmol of total CO_2—80 μmol in the air [ca. 3 liters] and liquid [ca. 500 ml] phases of the system plus 380 μmol that was added at the start of the experiment [see Materials and Methods] plus 1,000 μmol that must have been generated from Na_2CO_3 automatically pumped into the system [5] to neutralize the acid produced in this period from thiosulfate oxidation—which contained 10^8 cpm.) Thus, despite the fact that the direct calculation is based on very rough assumptions, it nevertheless comes to within 50% of accounting for CO_2 fixed in this culture. Similarly, in thiosulfate-biotin medium, where all the carbon must have come from CO_2, the counts taken up at $D = 0.02\ h^{-1}$ (54,900 cpm/mg of biomass per h per 10^8 cpm initially added) correspond to the assimilation of 5 μg carbon h^{-1}, again some 50% of the carbon actually assimilated in this culture (10 μg/h). We believe this to be a reasonable agreement considering the potentially wide margin of error in the various assumptions on which the direct quantitative calculations are based.

It is clear from the data that under certain nutrient-limited mixotrophic conditions, CO_2 contributes significantly to cell biosynthesis in *T. novellus*. Under such conditions, energy is derived concurrently from thiosulfate and glucose oxidations, as we showed in a previous study (5), and both glucose and CO_2 serve as significant sources of cell carbon, as we show here. Thus, a high degree of commingling of all facets of autotrophic and heterotrophic metabolisms occurs in *T. novellus* under these conditions. A similar commingling of the two types of physiologies appears to be present in *Thiobacillus* A2 under nutrient-limited mixotrophic conditions (3, 18).

Previous studies, using nutrient-excess conditions of batch culture, have shown that ribulose bisphosphate carboxylase is repressed in the presence of organic compounds in facultative chemolithotrophs (1, 6, 8, 9, 16), including *T. novellus* (1), and that in mixotrophic environments no significant assimilation of CO_2 takes place. The fact that under some of the nutrient-limited mixotrophic conditions used here CO_2 did serve as a significant source of cell carbon strongly suggests that the repression of ribulose bisphosphate carboxylase is mitigated when the organic compound is present at subsaturating levels. In this respect, this enzyme is similar to several other enzymes in this (5, 15) and other (8, 10) bacteria whose synthesis is repressed at high concentration of nutrients, but not when the same nutrients are present at subsaturating levels. We showed previously (5, 15) that *T.*

TABLE 2. Products of $^{14}CO_2$ assimilation excreted by steady-state cultures of *T. novellus*

Growth condition	Dilution rate (h^{-1})	^{14}C-containing organic material excreted in medium as % of total $^{14}CO_2$ fixed
Autotrophic	0.02	14 (15.9)[a]
(With 0.03% yeast extract)	0.03	5 (5.2)
	0.05	3 (2.8)
(With 10^{-5} biotin)	0.02	28 (39.4)
Heterotrophic	0.02	4 (0.5)
	0.03	3 (0.3)
	0.05	3 (0.4)
Mixotrophic	0.02	6. (1.1)
	0.03	8
	0.05[b]	4 (0.7)

[a] Numbers in parentheses represent amount of ^{14}C-containing products as percentage of total biomass synthesized. This was estimated from steady-state biomass under different conditions. An illustration of this calculation is as follows. At D = 0.02 h^{-1}, the steady-state biomass of the mixotrophic culture was 26 mg of cell material/100 ml of culture, and 17% of it came from CO_2 (Table 2), i.e., 4.4 mg/100 ml of culture. Since 6% of the total CO_2 fixed was excreted, this amount of cell material must have represented 94% of the total CO_2 fixed. Thus, 6% of the excreted CO_2 is equivalent to 0.28 mg of cell material/100 ml of culture, or 0.5% of the total biomass synthesized under these conditions.

[b] 3.33 mM glucose plus 15.17 mM $S_2O_3^{2-}$.

novellus possessed the regulatory resilience to make efficient and concurrent use of inorganic and organic energy substrates when they were in short supply, and yet to minimize energy generation from them when they were provided at a high concentration. The present study shows that this resilience extends also to the regulation of carbon metabolism in that CO_2, although eschewed as a carbon source in a nutrient-excess mixotrophic environment, is nevertheless made use of in a nutrient-limited mixotrophic environment.

What is the advantage to the organism of concurrent use of CO_2 and organic material in the synthesis of cellular material? Under conditions where the available inorganic energy source is vastly in excess of organic material, this advantage is self-evident: if the extant organic material is not sufficient to fulfill the biosynthetic demand generated by the available inorganic energy source, it makes excellent sense to make up the deficit by CO_2 fixation. However, in other situations, like one of the conditions examined here, this advantage is not as readily evident. We have seen that at a concentration of 15.17 mM $S_2O_3^{2-}$ and 1.67 mM glucose in the inflow medium, the organism opted to obtain a significant portion of its cell material from CO_2, even though the glucose provided was sufficient to completely fulfill the extant biosynthetic needs. The glucose thus spared was respired, leading to the curious situation that equivalent amounts of organic material were generated from CO_2 and respired back to CO_2. In energetic terms, and in terms of biomass synthesis, this is not a sensible strategy, since more energy has to be consumed to generate organic material from CO_2 than can be obtained from the complete oxidation of an equivalent amount of the organic material. However, this strategy may be advantageous as regards the growth rate that the organism can attain at these high $S_2O_3^{2-}$-to-glucose ratios under nutrient-limited conditions. The very minimal and subsaturating concentrations of glucose that can be expected to be present in steady-state cultures under these conditions would probably permit growth at a very low rate, and this could conceivabl be augmented by relying on CO_2 as a major carbon source, especially if, as was the case here, it is present at saturating levels. Since in nature selection appears to favor a faster-growing organism rather than a more efficient one, this strategy might be of survival value to a facultative chemolithotroph; the energy wasted in the process, especially when a larger flux of $S_2O_3^{2-}$ ensures its relative abundance, is probably well worth the price. These speculations are now being tested by studying competition between *T. novellus* and *T. perometabolis* under nutrient-limited mixotrophic conditions; *T. perometabolis* is a mixotroph like *T. novellus* but lacks the capacity to use CO_2 as a significant source of cell carbon (7).

Some of the CO_2 fixed was excreted into the medium by the organism as organic compounds of unknown identity under all conditions examined. In heterotrophic and mixotrophic media, this excretion was small—less than 1.1% of the total biomass synthesized. However, at D = 0.02 h^{-1} under autotrophic conditions, this excretion amounted to a full 16% of the biomass formed. This high amount of excretion probably occurred from the inability of the bacterium to adjust its CO_2-fixing machinery to lowered biosynthetic demand at this low rate of growth, a premise supported by the finding that at higher D values under these conditions, the excretion of organic material decreased drastically. For unknown reasons, when biotin replaced yeast extract in autotrophic medium, the excretion of organic substrates increased markedly. Excretion of organic material by obligate chemolithotrophs has been reported previously (2, 14, 17, 19), and Cohen et al. (2) have discussed its potential ecological implications. Since in *T.*

novellus significant excretion appears to occur only during autotrophic growth, which must be a rarity in the mixotrophic environments of nature, we believe that its ecological relevance is doubtful.

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