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Assimilation and Metabolism of Exogenous Organic Compounds by the Strict Autotrophs *Thiobacillus thioparus* and *Thiobacillus neapolitanus*

EMMETT J. JOHNSON[1] AND S. ABRAHAM

Bruce Lyon Memorial Research Laboratory, Children's Hospital Medical Center of Northern California, Oakland, California 94609

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The assimilation and utilization of the individual carbon atoms of pyruvate and acetate by cells of *Thiobacillus thioparus* and *T. neapolitanus*, in the presence and absence of an energy source, were studied by use of radioactive substrates. Both organisms produced $^{14}CO_2$ from ^{14}C-labeled pyruvate, but more came from carbon 1 than from carbons 2 or 3. The conversion of the carbons of acetate to CO_2 by both organisms was much less than that from any of the pyruvate carbons. When labeled pyruvate and acetate were incubated with these organisms, small amounts of radioactivity were found in the tricholoacetic acid-soluble material, nucleic acids, and lipids, and larger amounts were found in the protein fraction. The composition of the incubation medium affected the amount of utilization and incorporation of labeled substrates by both organisms. The presence of an exogenous energy source ($Na_2S_2O_3$) suppressed incorporation of the labeled substrates into various cellular components by *T. thioparus*, but enhanced incorporation by *T. neapolitanus*. When ^{14}C-pyruvate was used as a substrate, as many as 12 radioactive compounds were found in the water-soluble fraction in the experiments with *T. neapolitanus*, whereas no more than three radioactive compounds were detected in this fraction in the experiments with *T. thioparus*. Of the total ^{14}C activity found in the water-soluble fractions, malic acid contained the highest percentage. These findings are discussed in light of the overall metabolism of these two sulfur-oxidizing obligate chemoautotrophs, as well as in relation to the biochemical basis of chemoautotrophy.

One of the primary characteristics of autotrophic organisms is that they can satisfy all of their carbon requirement for growth and reproduction solely from carbon dioxide. To accomplish the reduction of CO_2 to the level of carbohydrate and other cellular components, both energy in the form of adenosine triphosphate (ATP) and reducing power in the form of reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) must be generated by the cell itself. The source of this energy in strict autotrophs such as the thiobacilli must be an inorganic substrate which, in this case, is usually some inorganic sulfur compound.

Obligate chemoautotrophic bacteria cannot grow on organic nutrients which support the growth of most heterotrophs (6, 8, 10, 13, 15). Their inability to grow heterotrophically is difficult to explain, since it is generally believed that their metabolism, aside from the production of ATP and reduced pyridine nucleotides through the oxidation of inorganic substrates and the fixation of CO_2, is likely to be similar in most respects to that of heterotrophs.

Until recently, evidence to support the view that obligate autotrophs can assimilate and metabolize organic compounds has been sparse. Recent work has shown that nitrifying autotrophs (7, 8, 10), as well as some thiobacilli (6, 13, 15), can incorporate a number of organic compounds into cell substance. The experiments reported here extend our knowledge of the metabolic capabilities and pathways of obligate autotrophs with respect to externally provided organic nutrients.

MATERIALS AND METHODS

Cultural procedures and preparation of cell suspensions. *Thiobacillus thioparus* (ATCC 8158) and *T.*

[1] Present address: Department of Microbiology, Tulane University School of Medicine, New Orleans, La. 70112.

neapolitanus (kindly provided by W. Vishniac) were grown in the medium of and according to the procedures described by Mayeux and Johnson (16). The cells were harvested by centrifugation at 2 to 4 C and were resuspended in (i) 0.025 M sodium phosphate buffer at *p*H 6.7 containing 3×10^{-3} M $MgCl_2$, (ii) the same phosphate buffer-$MgCl_2$ mixture containing 1% $Na_2S_2O_3$, or (iii) the complete growth medium (16). Cell suspensions were made with approximately 3 to 5 g of cells (wet weight) per 10 ml of the appropriate suspending medium. Samples of all cultures were plated on tryptose agar daily until harvested to check for heterotrophic contamination. These plates were incubated for 5 days at 30 C. None of the cultures used showed any heterotrophic contamination.

Procedure for incubation of cells with ^{14}C-labeled compounds. Incubations were carried out in 50-ml Erlenmeyer flasks fitted with glass center wells (11). A Beam Capsule (Ladd Research Industries, Burlington, Vt.) was placed inside each center well. All flasks contained, in the outside well, 1 ml of 0.025 M sodium phosphate buffer at *p*H 6.7 containing 3×10^{-3} M $MgCl_2$, the same phosphate buffer-$MgCl_2$ mixture containing 1% $Na_2S_2O_3$, or the complete medium (16), and pyruvate-*1*-^{14}C, pyruvate-*2*-^{14}C, pyruvate-*3*-^{14}C, acetate-*1*-^{14}C, or acetate-*2*-^{14}C as indicated in the legends of the tables. The reaction was initiated with 0.5 ml of the respective cell suspensions. The total volume in each flask was 1.5 ml. The flasks were sealed with self-sealing rubber serum caps and were incubated on a shaker water bath for 3 hr at 30 C in air. At the end of the incubation period, the stoppered flasks were immersed in a boiling-water bath for 30 sec to stop the reactions. After cooling, 0.3 ml of a 1:1 mixture of ethanolamine and 2-ethoxyethyl alcohol was injected into the Beam Capsule through the serum cap with a needle and syringe. Immediately thereafter, 0.5 ml of 0.1 N HCl was injected into the outside chamber of the flask to release the CO_2. The flasks were shaken at room temperature for 1 hr to insure complete absorption of the CO_2. The Beam Capsules were then removed and placed in a counting vial containing 10 ml of counting solution (see below for composition of the counting solution).

The contents of the outside well of the flask were separated by centrifugation and further fractionated according to the scheme given in Fig. 1.

Determination of pyruvate uptake. The aqueous phase was assayed spectrophotometrically with a commercial preparation of lactic dehydrogenase to determine the amount of unutilized pyruvate (2). The cuvettes contained the following: 37.5 mM glycylglycine buffer (*p*H 7.5), 0.125 mM NADH, 0.2 ml of sample, and an excess of crystalline lactic dehydrogenase (Calbiochem, Los Angeles, Calif.), all in a total volume of 2.0 ml. Changes in optical density were measured spectrophotometrically at 340 nm.

Chromatography and radioautography. Samples (0.05 ml) of the aqueous phase of each incubation mixture were chromatographed, in a descending manner, on Whatman no. 3 MM paper with a solvent system consisting of 100 ml of *n*-butyl alcohol, 22 ml of acetic acid, and 50 ml of water, for 18 hr at 20 to 25 C. Eight standards (alanine and glutamic, aspartic, citric, malic, lactic, succinic, and fumaric acids) were run on all chromatograms. Amino acid spots on chromatograms which were not radioautographed or eluted and counted were detected by spraying with ninhydrin (3). All chromatograms were sprayed with bromothymol blue (4 mg per 100 ml of 0.01 N NaOH) followed by exposure to ammonia vapors for detection of organic acids.

The chromatograms which were subjected to radioautography were not sprayed until after the radioautography. For radioautography, the chromatograms were exposed to Kodak medical X-ray film [14 by 17 inches (35.5 by 43.2 cm), single-coated, blue-sensitive] for 14 days.

The individual radioactive spots were located with the aid of the exposed X-ray film as a template and were cut from the chromatograms. Each spot was then cut into small pieces and placed in a single center-well flask. The compounds on the paper were combusted to CO_2, and the CO_2 was absorbed and assayed according to the procedure of Bartley and Abraham (1).

Other samples of the aqueous phase were spotted on strips of Whatman no. 3 MM paper and were chromatographed by ascending chromatography in the same solvent system as described above. The strips were then run through a strip counter (Nuclear-Chicago Actigraph II) to locate the radioactive areas.

Fractionation of lipids. The pellet of cellular material obtained from the initial centrifugation was washed once with 10 ml of glass-distilled water and centrifuged again; the supernatant fluid from the wash was discarded. The washed pellet was extracted with 5 ml of chloroform-methanol (2:1) in a water bath at 50 C for 5 min and then centrifuged. The residue was extracted three more times with 2 ml of the chloroform-methanol mixture each time, and the supernatant fluids (total lipid extract) were combined in an Erlenmeyer flask (125 ml) equipped with a side arm. The volume was reduced on a hot plate under a stream of nitrogen. The side arm was then filled with glass-distilled water, and the water was poured from the side arm into the main compartment of the flask. The mixture was then extracted five times with ligroin (boiling point, 30 to 60 C), and all of the extracted material was pooled in a 15-ml graduated centrifuge tube. (The flasks were carefully tipped with each extraction so that the water layer went into the side arm of the flasks and the ligroin was poured into the centrifuge tube.) The tubes were placed in a water bath at 50 C and the volumes were reduced to 2 ml under a stream of N_2. Duplicate samples of 0.2 ml each were placed in counting vials for determination of radioactivity in the total lipid fraction.

The remaining ligroin extract was evaporated to dryness and the residual lipids were treated with 1 ml of 15% KOH. A bubble stopper was placed on top of the centrifuge tube and the tube was heated at 80 C for 2 to 3 hr to saponify the lipids. The tube was cooled, and 0.5 ml of 95% ethyl alcohol was added to each tube followed by 3.0 ml of ligroin. The tube was stoppered with a black rubber stopper (previously

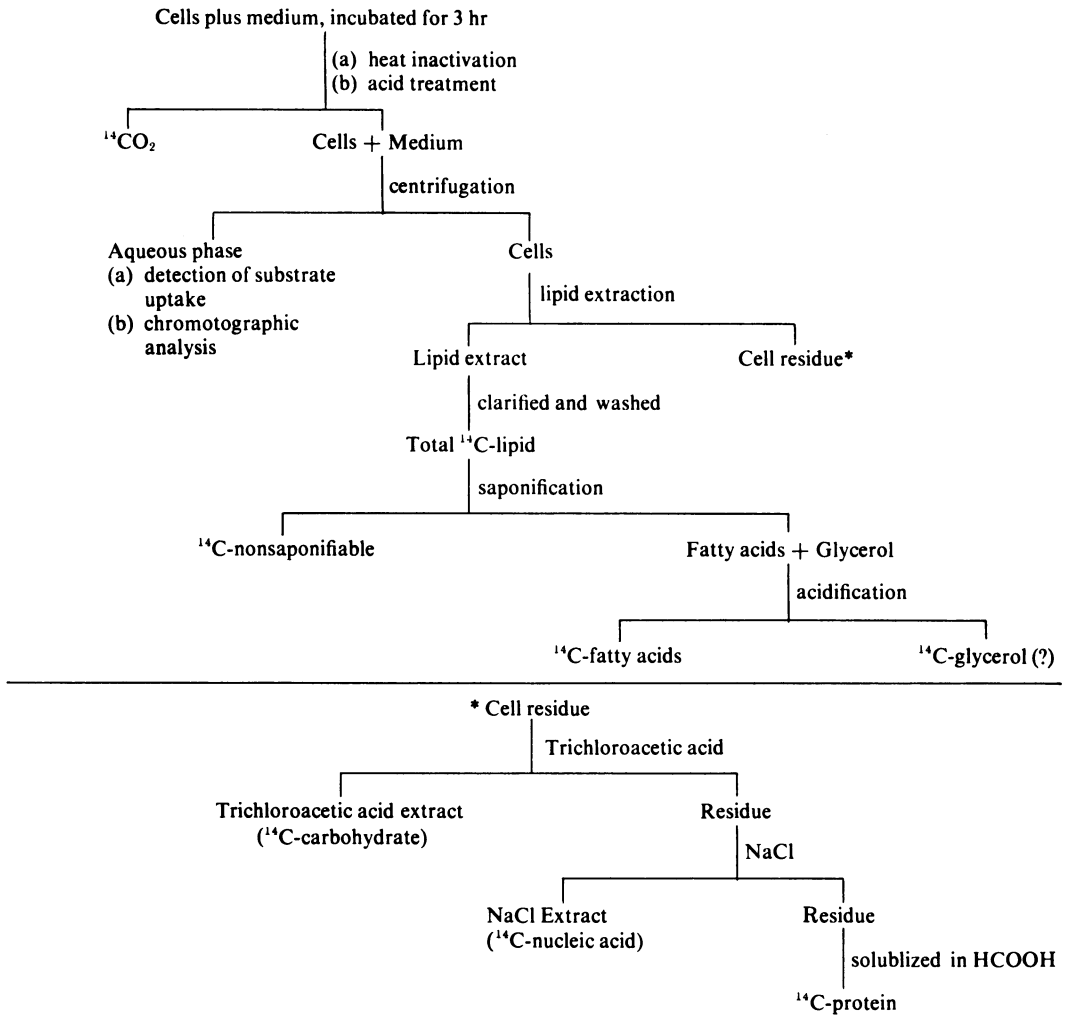

FIG. 1. *Fractionation of cells of T. thioparus and T. neapolitanus*

washed with acetone) and then was vigorously shaken. The phases were separated by centrifugation and the ligroin phase was removed with a Pasteur pipette and transferred to another centrifuge tube set in an ice bath. A second extraction with 2 ml of ligroin was performed, and the extracts were pooled in the tube in the ice bath. Duplicate samples (0.5 ml) of the pooled extract were placed in counting vials for the determination of radioactivity in the nonsaponifiable fraction.

The water residue of the saponification mixture was evaporated to one-half its volume and cooled in an ice bath; then 3 ml of hexane and 0.3 ml of concentrated HCl were added. The tubes were stoppered, shaken, and centrifuged. The hexane layer was removed with a Pasteur pipette and transferred to another tube. A second extraction with 2 ml of hexane was performed. The hexane fractions were pooled, and duplicate samples of 0.5 ml were placed in counting vials to determine the radioactivity in the fatty acids. Samples (0.05 ml) of the remaining aqueous fraction were placed in counting vials to determine radioactivity.

Preparation of cold trichloroacetic acid-soluble material. The defatted residue was extracted twice with 2 ml of 5% cold trichloroacetic acid. The trichloroacetic acid extracts were pooled, and a sample of 0.2 ml was used to determine radioactivity.

Preparation of the nucleic acid fraction. The residue from the extraction with cold trichloroacetic acid was treated with 1 ml of 10% NaCl at 80 C for 1 hr. A sample (0.05 ml) of this fraction and 0.15 ml of glass-distilled water were placed in a counting vial to determine the amount of radioactivity in the nucleic acid fraction (9).

Preparation of the protein fraction. The cell residue

from the sodium chloride extraction was washed with water and then dissolved in 1 ml of 88% formic acid. The protein was reprecipitated with 2 ml of 20% trichloroacetic acid and allowed to stand at 0 C for 1 hr. The precipitate was washed twice with 2 ml of glass-distilled water, once with ethyl alcohol-ether (3:1), and once with ether and then was allowed to dry in air. The dried precipitate was dissolved in 1 ml of 88% formic acid, and duplicate samples of 0.1 ml were placed in counting vials for the determination of the radioactivity in the protein fraction.

Radioactivity measurements. All quantitative measurements of radioactivity were made in 10 ml of a counting solution consisting of 5 g of 2,5-diphenyloxazole, 666 ml of toluene, and 333 ml of ethyleneglycol monomethyl ether. All determinations were made in an Ansitron Liquid Scintillation Spectrometer (Picker-Nuclear Corp., White Plains, N.Y.).

Chemicals, biochemicals, and radioactive compounds. All chemicals and solvents were reagent grade. Pyruvate-*1*-^{14}C, pyruvate-*2*-^{14}C, pyruvate-*3*-^{14}C, acetate-*1*-^{14}C, and acetate-*2*-^{14}C were purchased from New England Nuclear Corp., Boston, Mass. All pyruvate samples were stored in an equimolar solution of HCl and frozen as recommended by Von Korff (18) to assure stability.

RESULTS

The uptake of pyruvate, in each experiment, was measured in triplicate. For this purpose, samples from the flasks containing pyruvate-*1*-^{14}C, pyruvate-*2*-^{14}C, and pyruvate-*3*-^{14}C were taken under the various buffer conditions. Irrespective of the location of the labeled carbon, the uptake of pyruvate was shown to be the same for any given condition. In the experiments with *T. thioparus*, pyruvate uptake was 75, 60, and 60% in the phosphate buffer, phosphate plus thiosulfate buffer, and complete medium, respectively. In the experiments with *T. neapolitanus*, the uptake of pyruvate was 75, 70, and 75% in the phosphate buffer, phosphate buffer plus thiosulfate, and complete medium, respectively. (These values are based on the actual amounts of cellular protein incubated in each experiment and have not been adjusted per 10 mg of protein as were those of the metabolic data presented in the tables.)

Assimilation and metabolism of 1, 2, and 3-^{14}C-labeled pyruvate. The extent of conversion of the ^{14}C of pyruvate-*1*-^{14}C, pyruvate-*2*-^{14}C, and pyruvate-*3*-^{14}C to CO_2 by *T. thioparus* and *T. neapolitanus* is given in Table 1. Both organisms produced large amounts of CO_2 from pyruvate-*1*-^{14}C, 76% of the added pyruvate per 10 mg of cell protein in the case of *T. thioparus* and 44.8% in the case of *T. neapolitanus*, during the 3-hr incubation period when the cells were suspended

TABLE 1. *Incorporation of the various carbons of pyruvate into CO_2, trichloroacetic acid-soluble compounds, nucleic acids, and proteins by T. thioparus and T. neapolitanus*[a]

Labeled substrate	Incubation medium	Per cent of added isotope converted to							
		Carbon dioxide by		Trichloroacetic acid-soluble compounds by		Nucleic acids by		Proteins by	
		T. thioparus	*T. neapolitanus*	*T. thioparus*	*T. neapolitanus*	*T. thioparus*	*T. neapolitanus*	*T. thioparus*	*T. neapolitanus*
Pyruvate-*1*-^{14}C	PO_4^{3-}	76.16	44.81	0.56	0.13	0.64	0.02	7.22	0.10
	PO_4^{3-} + $S_2O_3^{2-}$	13.79	18.88	0.28	0.09	0.27	0.20	1.86	1.25
	Complete	14.49	33.07	0.05	0.12	0.08	0.13	0.85	1.45
Pyruvate-*2*-^{14}C	PO_4^{3-}	30.76	0.46	0.80	0.14	0.80	0.03	11.50	0.24
	PO_4^{3-} + $S_2O_3^{2-}$	3.92	0.35	0.19	0.12	0.16	0.07	2.21	0.97
	Complete	3.18	1.03	0.12	0.18	0.06	0.14	0.86	1.48
Pyruvate-*3*-^{14}C	PO_4^{3-}	34.92	0.40	0.95	0.23	0.86	0.03	12.87	0.14
	PO_4^{3-} + $S_2O_3^{2-}$	7.29	0.59	0.13	0.12	0.17	0.09	1.93	1.34
	Complete	2.41	1.59	0.19	0.25	0.03	0.18	1.00	1.47

[a] Unless otherwise specified, the complete system contained: 25 μmoles of sodium phosphate buffer (PO_4^{3-}; *p*H 6.7), 3 μmoles of $MgCl_2$, 1 μmole of pyruvate-*1*-^{14}C, -*2*-^{14}C, or -*3*-^{14}C (approximately 5×10^5 counts min), and 0.5 ml of the cell suspension in a total volume of 1.5 ml. Cell protein added to the incubation flasks was as follows: for *T. thioparus*, 3.1, 5.3, and 5.7 mg for PO_4^{3-} buffer, PO_4^{3-} buffer + $S_2O_3^{2-}$, and the complete growth medium (20), respectively; and for *T. neapolitanus*, 6.0, 7.6, and 7.1 mg for PO_4^{3-} buffer, PO_4^{3-} + $S_2O_3^{2-}$, and the complete growth medium (16), respectively. The incubations were carried out for 3 hr with mechanical shaking at 30 C with air as the gas phase. All values recorded were calculated per 10 mg of cell protein and are averages of duplicate determinations.

in phosphate buffer. The presence of an energy source ($Na_2S_2O_3$) or the components of the complete growth medium suppressed CO_2 production from pyruvate-*1*-^{14}C in both organisms and from pyruvate-*2*-^{14}C and pyruvate-*3*-^{14}C in *T. thioparus*. Both organisms produced much less CO_2 from pyruvate-*2*-^{14}C and pyruvate-*3*-^{14}C than from pyruvate-*1*-^{14}C. In the case of *T. neapolitanus*, very little $^{14}CO_2$ was produced from pyruvate-*2*-^{14}C or pyruvate-*3*-^{14}C under any of the incubation conditions employed.

There was significant incorporation of all of the labeled pyruvates into trichloroacetic acid-soluble compounds, nucleic acids, and proteins by *T. thioparus* (Table 1); however, in the presence of thiosulfate or the complete medium, incorporation into these compounds was repressed or inhibited. The largest amount of incorporation occurred in the protein fraction.

The incorporation of all of the labeled pyruvates into trichloroacetic acid-soluble compounds, nucleic acids, and proteins by *T. neapolitanus* was much lower than by *T. thioparus* (Table 1). However, in the case of *T. neapolitanus*, in the presence of thiosulphate or the complete medium, there was a stimulation of incorporation into the nucleic acid and protein fractions but not into the trichloroacetic acid-soluble compounds.

Cells of *T. thioparus* incorporated small amounts of pyruvate-*1*-^{14}C into the total lipid fraction when suspended in phosphate buffer (Table 2), but no significant amount of incorporation occurred when the cells were suspended in either phosphate buffer plus $Na_2S_2O_3$ or in the complete medium. There was more incorporation of pyruvate-*2*-^{14}C and pyruvate-*3*-^{14}C than pyruvate-*1*-^{14}C into lipids by cells of *T. thioparus* suspended in phosphate buffer. Again, there was no significant incorporation when the cells were suspended in either phosphate buffer plus $Na_2S_2O_3$ or in the complete medium.

Cells of *T. neapolitanus* incorporated much less of any of the labeled pyruvates into lipids than did *T. thioparus* under any of the conditions tested. The presence of an inorganic energy source increased the extent of incorporation. Although there was some incorporation into nonsaponifiable lipids, fatty acids, and glycerol, there was much greater incorporation into fatty acids than into any of the other fractions in all cases with both organisms.

Assimilation and metabolism of 1- and 2-^{14}C-labeled acetate. The amount of conversion of acetate-*1*-^{14}C and acetate-*2*-^{14}C to CO_2 by *T. thioparus* and *T. neapolitanus* is shown in Table 3. Cells of *T. thioparus* suspended in phosphate buffer converted 11% of the added acetate-*1*-^{14}C and 3% of the added acetate-*2*-^{14}C to CO_2 during the 3-hr incubation period. As was the case with the labeled pyruvates (Table 1), less CO_2 was produced when the cells were suspended in phosphate buffer plus $Na_2S_2O_3$ or in the complete medium.

Essentially no CO_2 was produced from eithe

TABLE 2. *Incorporation of the ^{14}C of pyruvate-1-^{14}C, pyruvate-2-^{14}C, and pyruvate-3-^{14}C into lipids by T. thioparus and T. neapolitanus*[a]

Organism	Per cent of added isotope incorporated into lipid fractions from								
	Pyruvate-*1*-^{14}C			Pyruvate-*2*-^{14}C			Pyruvate-*3*-^{14}C		
	PO_4^{3-} buffer	PO_4^{3-} buffer + $S_2O_3^{2-}$	Complete medium	PO_4^{3-} buffer	PO_4^{3-} Buffer + $S_2O_3^{2-}$	Complete medium	PO_4^{3-} buffer	PO_4^{3-} buffer + $S_2O_3^{2-}$	Complete medium
T. thioparus									
Total lipids	0.26	0.07	0.03	3.10	0.02	0.03	4.1	0.02	0.15
Nonsaponifiable lipids				0.63			0.88		0.04
Fatty acids				2.18			2.63		0.05
Glycerol				0.19			0.17		0.08
T. neapolitanus									
Total lipids	0.13	1.14	0.16	0.06	0.67	0.47	0.08	0.70	0.41
Nonsaponifiable lipids	0.05	0.02	0.02		0.08	0.16		0.14	0.06
Fatty acids		0.06			0.30	0.09		0.34	0.19
Glycerol	0.09	0.10	0.10		0.09			0.16	0.12

[a] See Table 1 for experimental details.

TABLE 3. *Incorporation of the various carbons of acetate into CO_2, trichloroacetic acid-soluble compounds, nucleic acids, and proteins by T. thioparus and T. neapolitanus*[a]

Labeled substrate	Incubation medium	Per cent of added isotope converted to							
		Carbon dioxide by		Trichloroacetic acid-soluble compounds by		Nucleic acids by		Proteins by	
		T. thioparus	*T. neapolitanus*	*T. thioparus*	*T. neapolitanus*	*T. thioparus*	*T. neapolitanus*	*T. thioparus*	*T. neapolitanus*
Acetate-*1*-^{14}C	PO_4^{3-}	11.11	0.02	0.18	0.01	0.15	0.01	4.60	0.04
	PO_4^{3-} + $S_2O_3^{2-}$	1.05	0.04	0.04	0.04	0.02	0.01	0.36	0.21
	Complete	2.34	0.01	0.04	0.02	0.03	0	0.45	0.19
Acetate-*2*-^{14}C	PO_4^{3-}	3.29	0.03	0.28	0.01	0.18	0.01	4.82	0.05
	PO_4^{3-} + $S_2O_3^{2-}$	0.34	0	0.04	0.02	0.04	0	0.50	0.22
	Complete	0.28	0.01	0.04	0.02	0.02	0	0.52	0.21

[a] Experimental conditions are given in Table 1, except that the ^{14}C-labeled pyruvates were substituted for by 1 μmole of acetate-*1*-^{14}C and acetate-*2*-^{14}C (approximately 4×10^6 counts/min).

of the labeled acetates by cells of *T. neapolitanus* under any of the conditions tested.

Small but significant amounts of both of the labeled acetates were incorporated into trichloroacetic acid-soluble compounds, nucleic acids, and proteins by *T. thioparus* (Table 3). As was the case with the incorporation of the labeled pyruvates, in the presence of thiosulphate or the complete medium, incorporation into these compounds was repressed or inhibited. Again, the largest amount of incorporation occurred in the protein fraction.

There was essentially no incorporation of either of the labeled acetates into trichloroacetic acid-soluble compounds or nucleic acids by *T. neapolitanus* under any of the conditions tested (Table 3). A small but significant amount of incorporation of the labeled acetates into protein occurred in the experiments with *T. neapolitanus*, but only in the presence of an energy source.

There was approximately the same amount of incorporation of acetate-*1*-^{14}C and acetate-*2*-^{14}C into lipids by cells of *T. thioparus* for any given conditions (Table 4). More radioactivity was observed in this fraction when the cells were suspended in phosphate buffer alone than when they were suspended in phosphate buffer plus $Na_2S_2O_3$ or in the complete medium. Cells of *T. neapolitanus* also incorporated approximately the same amount of acetate-*1*-^{14}C and acetate-*2*-^{14}C into lipids for any given conditions. Significant incorporation occurred only in the presence of an inorganic energy source. There was more incorporation into the fatty acid fraction than into any other fraction by both organisms.

Intermediates in the metabolism of pyruvate. Chromatography and radioautography of the aqueous supernatant fluids from systems with *T. thioparus* revealed three radioactive areas derived from the metabolism of pyruvate-*1*-^{14}C, pyruvate-*2*-^{14}C, and pyruvate-*3*-^{14}C when the cells were suspended in phosphate buffer (Fig. 2). These three spots were glutamic and malic acids and alanine. The same three spots appeared with all species of ^{14}C-labeled pyruvate when the cells were suspended in the complete growth medium, but no labeled alanine was detected when the cells were suspended in phosphate buffer plus $Na_2S_2O_3$.

As many as 12 radioactive compounds were detected in the aqueous supernatant fluid from systems with *T. neapolitanus* when the cells were metabolizing pyruvate-*2*-^{14}C and pyruvate-*3*-^{14}C in phosphate buffer with $Na_2S_2O_3$ (Fig. 3). Seven of these spots were identified as aspartic, glutamic, citric, malic, and fumaric acids and alanine. One other spot was located in the area where lactic and succinic acids overlap on the chromatograms, but it occupied a position almost exactly adjacent to known lactic acid. The five other spots have not been identified. Under the same conditions, no labeled glutamic or lactic acid was detected when pyruvate-*1*-^{14}C was the substrate (Fig. 3).

A similar pattern occurred when the cells were suspended in the complete growth medium. When pyruvate-*1*-^{14}C was used, no ^{14}C-alanine or ^{14}C-lactic acid was detected.

When cells of *T. neapolitanus* were incubated in phosphate buffer, the chromatograms and radioautograms revealed the same radioactive spots with either pyruvate-*1*-^{14}C, pyruvate-*2*-^{14}C, or

TABLE 4. *Incorporation of the ^{14}C of acetate-1-^{14}C and acetate-2-^{14}C into lipids by T. thioparus and T. neapolitanus*[a]

Organism	Per cent of added isotope incorporated into lipid fractions from					
	Acetate-1-^{14}C			Acetate-2-^{14}C		
	PO_4^{3-} buffer	PO_4^{3-} buffer + $S_2O_3^{2-}$	Complete medium	PO_4^{3-} buffer	PO_4^{3-} Buffer + $S_2O_3^{2-}$	Complete medium
T. thioparus						
Total lipids	3.76	0.11	0.12	4.42	0.45	0.25
Nonsaponifiable lipids	0.82	0.03	0.03	0.88	0.08	0.05
Fatty acids	2.67	0.07	0.09	2.93	0.31	0.15
Glycerol	0.06	0	0.01	0.08	0.03	0.03
T. neapolitanus						
Total lipids	0.01	0.48	0.17	0	0.45	0.13
Nonsaponifiable lipids		0.09	0.03	0	0.08	0.02
Fatty acids		0.30	0.11	0	0.27	0.11
Glycerol		0.04	0.05	0	0.07	0.02

[a] See Tables 1 and 3 for experimental details.

pyruvate-3-^{14}C. Not all 12 spots were detected. Two of the unidentified spots, the third and fourth from the origin, and fumaric acid were not detected under these conditions.

Strip-counting analysis as well as combustion of the radioactive areas found on paper chromatograms showed that the largest amount of radioactivity originating from pyruvate appeared in malic acid (Table 5). In the case of *T. thioparus*, the maximal incorporation of pyruvate carbon into malic acid (18.2%) occurred in the experiments with phosphate buffer plus $Na_2S_2O_3$ when pyruvate-3-^{14}C served as the labeled substrate. On the other hand, in the experiments with *T. neapolitanus*, the highest incorporation (10.9%) of the third carbon of pyruvate into malic acid occurred when the incubations were performed in phosphate buffer alone. With phosphate buffer plus $Na_2S_2O_3$ or with the complete medium, only 1% or less of the added ^{14}C was recovered in malic acid. At present, the reason for this is not clear. Much smaller percentages of added ^{14}C from the ^{14}C-labeled pyruvates occurred in all other compounds identified on the chromatograms (Table 6).

DISCUSSION

In recent years, there has been renewed interest in the exact meaning of obligate autotrophy (8, 10, 13–15). It is clear from the work of several investigators that obligate autotrophs generally cannot grow heterotrophically (6, 8, 10, 13, 15). *T. thiooxidans* has been grown on glucose under very special conditions (5), but whether this can be achieved with other obligate autotrophs is not known. It is equally clear from the work of a number of investigators that obligate autotrophs are capable of assimilating and metabolizing organic compounds (6–8, 10, 13, 15). There are differences, however, in the metabolism of different organic compounds by different obligate autotrophs. We have presented comparative data for two organisms which are generally considered to be closely related species of the genus *Thiobacillus*, namely *T. thioparus* and *T. neapolitanus*. It is clear that their responses to the presence of organic compounds under a variety of incubation conditions are quite different.

More CO_2 was produced from pyruvate-1-^{14}C, pyruvate-2-^{14}C, and pyruvate-3-^{14}C by *T. thioparus* than by *T. neapolitanus* when the cells were suspended in phosphate buffer. The difference was especially striking when pyruvate-2-^{14}C and pyruvate-3-^{14}C served as labeled substrate. This finding suggests a greater capacity for randomization of pyruvate carbon by *T. thioparus* than by *T. neapolitanus*.

In all instances with *T. thioparus*, the presence of $Na_2S_2O_3$ suppressed the amount of CO_2 produced from any of the labeled pyruvates. The same situation prevailed with *T. neapolitanus*, but only with pyruvate-1-^{14}C. The meaning of this is not clear.

One might explain the suppression of CO_2 production by thiosulfate by suggesting a sparing effect; i.e., when the cells have no energy source, a greater demand is placed on them to use any available exogenous organic compound for en-

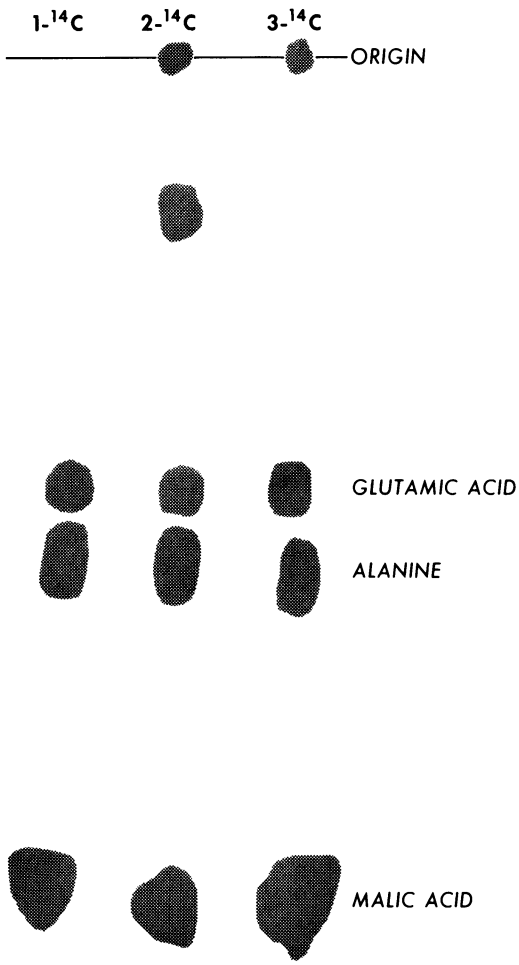

FIG. 2. *Chromatographic analysis of water-soluble compounds derived from carbons 1, 2, and 3 of pyruvate produced by cells of T. thioparus incubated in phosphate buffer. Spots indicated above represent those compounds which had ^{14}C activity as determined by radioautography. The identification of each spot, where known, is also shown above.*

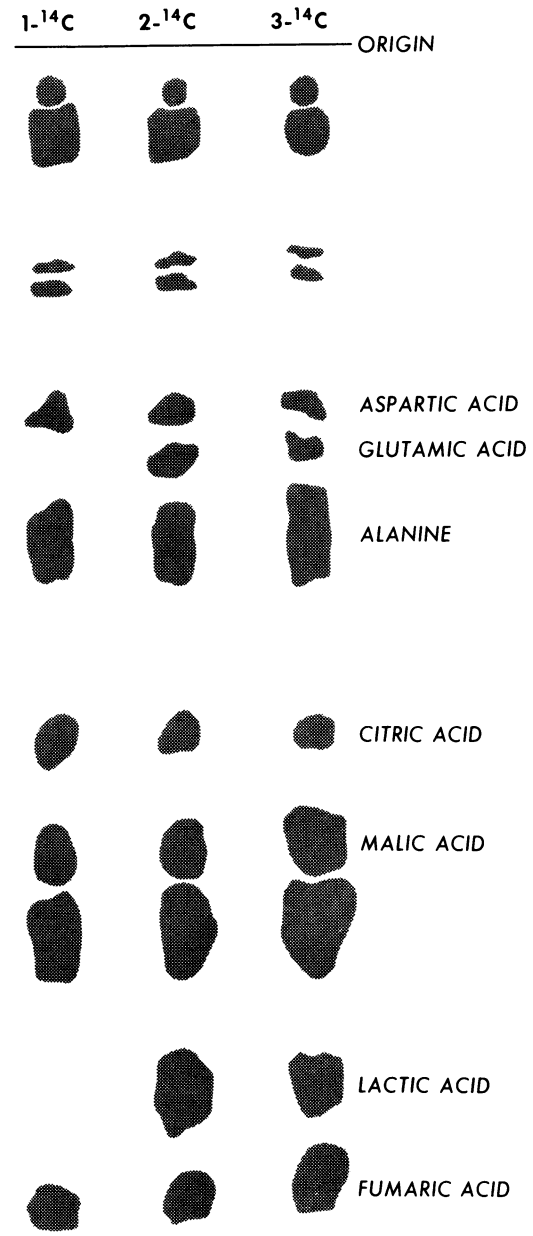

FIG. 3. *Chromatographic analysis of water-soluble compounds derived from carbons 1, 2, and 3 of pyruvate produced by cells of T. neapolitanus incubated in phosphate buffer plus added thiosulfate. Spots indicated above represent those compounds which had ^{14}C activity as determined by radioautography. The identification of each spot, where known, is also shown above.*

ergy, whereas the presence of their preferred energy source, some inorganic compound of sulfur, relieves this demand and therefore less of the organic compound is metabolized. The apparent failure of this pattern of utilization to appear in the case of pyruvate-*2-^{14}C* and pyruvate-*3-^{14}C* with cells of *T. neapolitanus* may result from the difficulty in detecting differences at this low level of oxidation.

One difficulty with the aforementioned energy-sparing hypothesis, however, is that cells of *T. thioparus* suspended in phosphate buffer, especially after one washing, contain appreciable amounts of inorganic sulfur and therefore would presumably have an available inorganic energy source. After one washing, cells of *T. neapolitanus*, on the other hand, contain little or no detectable inorganic sulfur.

TABLE 5. *Conversion of pyruvate-1-^{14}C, -2-^{14}C, and -3-^{14}C into malic acid by T. thioparus and T. neapolitanus*[a]

Organism	Per cent of added isotope converted to malic acid from								
	Pyruvate-1-^{14}C			Pyruvate-2-^{14}C			Pyruvate-3-^{14}C		
	PO_4^{3-} buffer	PO_4^{3-} buffer + $S_2O_3^{2-}$	Complete medium	PO_4^{3-} buffer	PO_4^{3-} buffer + $S_2O_3^{2-}$	Complete medium	PO_4^{3-} buffer	PO_4^{3-} buffer + $S_2O_3^{2-}$	Complete medium
T. thioparus	12.9	10.3	4.5	14.8	17.0	7.8	14.7	18.2	11.9
T. neapolitanus	5.9	1.1	0.1	10.9	2.0	0.3	10.9	1.5	0.3

[a] See Table 1 for experimental details. Malic acid was isolated from the aqueous phase by paper chromatography and assayed for ^{14}C activity.

TABLE 6. *Conversion of pyruvate-1-^{14}C, -2-^{14}C, and -3-^{14}C into various organic and amino acids by T. thioparus and T. neapolitanus*[a]

Labeled substrate	Incubation medium	Per cent of added isotope converted to							
		Glutamate by		Alanine by		Aspartate by *T. neapolitanus*	Citrate by *T. neapolitanus*	Fumarate by *T. neapolitanus*	Lactate by *T. neapolitanus*
		T. thioparus	*T. neapolitanus*	*T. thioparus*	*T. neapolitanus*				
Pyruvate-1-^{14}C	PO_4^{3-}	0.8	0.2	1.7	1.2	1.0	0.2		
	PO_4^{3-} + $S_2O_3^{2-}$	1.1			1.8	0.3	0.2	0.3	
	Complete	1.3	1.4	1.7			0.1	0.4	
Pyruvate-2-^{14}C	PO_4^{3-}	1.9	0.5	4.7	0.8	0.9	0.4		
	PO_4^{3-} + $S_2O_3^{2-}$	8.7	0.8		1.3	0.3	0.4	0.5	1.1
	Complete	6.8	1.9	1.3	0.7		0.1	0.1	0.3
Pyruvate-3-^{14}C	PO_4^{3-}	2.0	0.5	4.9	2.3	0.8	0.7		
	PO_4^{3-} + $S_2O_3^{2-}$	10.1	1.2		1.9	0.3	0.4	1.1	1.1
	Complete	11.1	lost	0.6	0.2		0.3	0.5	1.1

[a] See Table 1 for experimental details. The organic and amino acids were isolated from the aqueous phase by paper chromatography and assayed for ^{14}C activity.

The difference between the production of CO_2 from labeled acetate and pyruvate by the two organisms suggests either that the cells are less permeable to acetate than to pyruvate, which would be in contrast to the findings of Ida and Alexander with *Nitrobacter agilis* (10), or alternatively that there is no mechanism for the direct utilization of acetate in *T. neapolitanus* and only low levels of the required enzymes in *T. thioparus*. More work is required to distinguish between these two possibilities.

It is clear that small but significant amounts of label from pyruvate were incorporated into cold trichloroacetic acid-soluble material and nucleic acids by both organisms, but more was incorporated in *T. thioparus* than in *T. neapolitanus*. There was incorporation of acetate-1-^{14}C and acetate-2-^{14}C into cold trichloroacetic acid-soluble material and nucleic acids by *T. thioparus* when the cells were suspended in phosphate buffer.

The pattern of incorporation of pyruvate-2-^{14}C and pyruvate-3-^{14}C into lipids is almost identical with that for acetate-1-^{14}C and acetate-2-^{14}C, particularly in the case of *T. thioparus*. It is clear from these results that the 2-carbon product derived from the decarboxylation of pyruvate is being incorporated into lipid as a unit as is the externally provided acetate. At the present time, it is not clear why $Na_2S_2O_3$ suppresses this incorporation in *T. thioparus*. It appears that the carry-over sulfur provides an adequate energy source for lipid synthesis and that $Na_2S_2O_3$ antagonizes this activity. In the case of *T. neapolitanus*, where there is little or no carry-over sulfur, the requirement for an energy source in lipid synthesis is quite evident.

Cells of *T. thioparus* incubated in phosphate buffer incorporated relatively large amounts of all of the labeled pyruvates and acetates into protein, but again the presence of $Na_2S_2O_3$ decreased

such incorporation. Thus, it appears that carry-over sulfur provides an adequate energy source for protein and lipid synthesis and that $Na_2S_2O_3$ also antagonizes these activities.

It cannot be overlooked that *T. thioparus* is less acid-tolerant than *T. neapolitanus*. It is possible that in the presence of thiosulfate the *p*H drops considerably due to acid production which is inhibitory to *T. thioparus* but not to *T. neapolitanus*; however, one piece of evidence which argues against this is that CO_2 production from pyruvate-*1*-^{14}C by both organisms is inhibited by thiosulfate. This suggests that, if lowering of the *p*H is responsible for the inhibition by thiosulfate, then *T. neapolitanus* would be acid-tolerant with respect to the incorporation activities but not with respect to CO_2 production.

Cells of *T. neapolitanus* incorporated less of the labeled pyruvates and acetates into protein than did cells of *T. thioparus*. However, since washed cells of *T. neapolitanus* are essentially sulfur-free, it is clear that an energy source is required for protein synthesis since only in the presence of $Na_2S_2O_3$ or of the complete growth medium did incorporation occur. Kelly (12) also found that the assimilation of acetate-*2*-^{14}C was thiosulfate-dependent in *T. neapolitanus*.

There is a striking difference in the labeling pattern of the water-soluble compounds from *T. thioparus* and from *T. neapolitanus* when metabolizing labeled pyruvate. There were only three radioactive compounds detectable in this fraction from *T. thioparus*, whereas there were as many as 12 radioactive compounds in this fraction from *T. neapolitanus*. The production of labeled citric, malic, and fumaric acids by *T. neapolitanus* might suggest a nonfunctioning citric acid cycle. Evidence for a nonfunctioning citric acid cycle in *T. thioparus* and *T. thiooxidans* has been presented (17), but the equivalent evidence for *T. neapolitanus* has not. For ^{14}C-malic acid to accumulate from ^{14}C-pyruvate, as was the case in these experiments, both organisms would have to have either a complete citric acid cycle or a pyruvic carboxylase. Both organisms are known to have phosphoenolpyruvate carboxylase, but it is not known whether they have a pyruvate carboxylase. It is also not known whether they lack α-ketoglutaric dehydrogenase, as reported for another strain of *T. thioparus* and *T. thiooxidans* (21). A detailed study of the enzymes requisite to any given cycle would be necessary before any sound conclusions can be made about metabolic pathways. A separate study of a number of these enzymes has been made and will be the subject of a separate report.

Pyruvate appears to be converted directly to alanine. Additional evidence of transaminase activity or direct amination was found in the appearance of ^{14}C in both glutamic and aspartic acids.

Recently, a number of investigators have made efforts to understand the basis of obligate autotrophy (6, 8, 10, 13–15). Most authors present approximately the same list of possible factors responsible for obligate autotrophy (for enumeration see Kelly, reference 14, p. 43). One point needs emphasis, namely, that although all of the organisms involved in the many investigations may have obligate autotrophy in common, they may not necessarily have the same basis for this obligate autotrophy. The available evidence does not allow the adoption of any single hypothesis as adequate to explain the evolution of all obligate autotrophs.

The information provided previously by a number of investigators (4–8, 10, 13–14) and the information in this report aid in furthering our understanding of chemoautotrophic life and the ability of these organisms to assimilate and utilize organic compounds. In particular, these data point up the differences in the metabolism of these two supposedly closely related species of the genus *Thiobacillus*.

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