Evidence for the Calvin Cycle and Hexose Monophosphate Pathway in *Thiobacillus ferrooxidans*¹

NORD L. GALE² AND JAY V. BECK

Department of Bacteriology, Brigham Young University, Provo, Utah 84601

Received for publication 7 July 1967

The enzymes of the Calvin reductive pentose phosphate cycle and the hexose monophosphate pathway have been demonstrated in cell-free extracts of *Thiobacillus ferrooxidans*. This, together with analyses of the products of CO₂ fixation in cell-free systems, suggests that these pathways are operative in whole cells of this microorganism. Nevertheless, the amount of CO₂ fixed in these cell-free systems was limited by the type and amount of compound added as substrate. The inability of cell extracts to regenerate pentose phosphates and to perpetuate the cyclic fixation of CO₂ is partially attributable to low activity of triose phosphate dehydrogenase under the experimental conditions found to be optimal for the enzymes involved in the utilization of ribose-5-phosphate or ribulose-1,5-diphosphate as substrate for CO₂ incorporation. With the exception of ribulose-1,5-diphosphate, all substrates required the addition of adenosine triphosphate (ATP) or adenosine diphosphate (ADP) for CO₂ fixation. Under optimal conditions, with ribose-5-phosphate serving as substrate, each micromole of ATP added resulted in the fixation of 1.5 μmoles of CO₂, whereas each micromole of ADP resulted in 0.5 μmole of CO₂ fixed. These values reflect the activity of adenylate kinase in the extract preparations. The *K_m* for ATP in the phosphoribulokinase reaction was 0.91×10^{-3} M. Kinetic studies conducted with carboxydismutase showed *K_m* values of 1.15×10^{-4} M and 5×10^{-2} M for ribulose-1,5-diphosphate and bicarbonate, respectively.

The reductive pentose phosphate or Calvin pathway for the fixation of atmospheric carbon dioxide has been demonstrated or implicated in a number of chemoautotrophic bacteria, including *Thiobacillus thioparus* (24), several hydrogen bacteria (30; B. A. McFadden and C. L. Tu, Bacteriol. Proc., p. 94, 1966), *T. denitrificans* (20, 27, 28), *T. thiooxidans* (25), *T. novellus* (2), and *Nitrobacter agilis* (1). In addition, chromatographic studies of extracts of cells of the iron-oxidizing *Ferrobacillus ferrooxidans* grown on ¹⁴C- and ³²P-labeled media indicated that the latter microorganism was no exception. It was proposed, on the basis of such product analysis, that the Calvin cycle was operative in whole cells of the ferrobacilli (19).

Chemoautotrophic bacteria capable of oxidiz-

ing ferrous iron have been isolated from a number of geographical locations, in close association with the acidic leaching water from copper mines (5, 8, 9) or from bituminous coal mines (10, 16, 18, 26). Although the literature refers to iron-oxidizing isolates by various names—*T. ferrooxidans*, *F. ferrooxidans*, or *F. sulfooxidans*—all are quite similar, and it would appear, upon consideration of the descriptions given, that the cultures isolated from the several geographical locations are probably identical. It has been suggested (6, 14, 15) that the most appropriate name for all iron-oxidizing eubacteria which also oxidize some form of sulfur is *T. ferrooxidans*.

This work shows that the Calvin cycle, as well as the hexose monophosphate pathway, are operative in cell-free extracts of the microorganisms described by Beck (5) and identified as *T. ferrooxidans*. In 1965, K. J. Andersen and D. G. Lundgren (Bacteriol. Proc., p. 83) reported the presence of the enzymes involved in normal glycolysis and the Krebs cycle in extracts of *F.*

¹ This work was taken from a dissertation by Nord L. Gale in partial fulfillment of the requirements for the Ph.D. degree.

² Present address: Bio-Sciences Division of Physical Research Center, TRW Systems, Redondo Beach, Calif.

ferrooxidans, but they could not detect the enzymes of the hexose monophosphate pathway.

MATERIALS AND METHODS

Cell growth. *T. ferrooxidans* cells were grown in continuous culture as described by Beck and Shafia (6), and harvested with a Sharples centrifuge. The cells were separated from insoluble ferric compounds by differential centrifugation and were washed at least twice with and were suspended and stored in 0.01 N sulfuric acid at 2 to 4 C. Prior to preparation of extracts, the cells were washed three times with distilled water.

Extract preparation. Of washed cells, 6 to 10 g (wet weight) was suspended in 20 ml of distilled water with approximately 5 g of powdered glass (200-mesh) and was subjected to sonic oscillation at the maximal output of the model S75 Branson Sonifier for 5 to 7 min. Temperature was maintained below 4 C by holding the stainless-steel container in an ice-salt water bath during sonic treatment. The resultant extract was used either immediately or after centrifugation for 20 min at $20,000 \times g$ to remove whole cells, glass powder, and debris. Better breakage of cells and higher resultant protein content of the extracts could be insured by prior treatment of the cleaned, washed cells with approximately 3 g each of IR 45 and IR 120 (Amberlite; Rohm & Haas, Co., Philadelphia, Pa.) ion-exchange resins (23). The suspension of cells and added anion- and cation-exchange resins was shaken vigorously at ice bath temperatures for 10 min. The resins were allowed to settle, after which the cell suspension was decanted off into a separate container. The resins were washed two or three times with distilled water and the washings were combined. After centrifugation and resuspension in 20 ml of distilled water, the cells, together with added glass powder, were subjected to sonic treatment as described above. Some of the extracts used in this study were dialyzed in the cold against a 1-liter volume of 0.03 M tris(hydroxymethyl)aminomethane (Tris) chloride (pH 8.0) containing 10^{-4} M ethylenediaminetetraacetic acid (EDTA) and 5×10^{-4} M reduced glutathione (GSH). The bath was changed once during the 18-hr procedure. Dialysis of crude extracts did not significantly alter their behavior in the types of experiments performed in this work.

Protein determination. Protein concentration in the crude extracts was determined after centrifugation to remove debris and whole cells by comparing the ratios of the ultraviolet absorbancies at 280 and 260 m μ , according to the method of Warburg and Christian (31).

Optical density (OD) measurements were made on either a Beckman model DU or a Cary model 15 recording spectrophotometer.

Enzyme assays. Ribulose diphosphate carboxydismutase was measured by incubating cell-free extracts with Tris chloride buffer, MgCl₂, ¹⁴C-labeled sodium bicarbonate, and ribulose-1,5-diphosphate (RuDP; Sigma Chemical Co., St. Louis, Mo.). The reactants were placed in a 5-ml vaccine vial, quickly

flushed with nitrogen gas, capped, and placed in a Dubnoff metabolic shaking incubator at 30 C. Samples of 0.3 to 0.5 ml were taken at desired intervals by tuberculin syringes. The samples were placed in stainless-steel planchettes, acidified by the addition of 0.1 ml of 6 N HCl, and dried under an infrared heat lamp. Acid-stable products of fixation were counted on a thin window flow counter (Tracerlab, Richmond, Calif.).

Pentose phosphate isomerase and phosphoribulokinase were conveniently assayed by replacing RuDP by ribose-5-phosphate (R-5-P) and adenosine-5'-triphosphate (ATP) in the above reaction mixture. In the presence of limiting concentrations of ATP, the phosphoribulokinase activity became the rate-limiting step in the fixation of CO₂, thus permitting kinetic studies to be made on this critical enzyme. A detailed study of this activity in extracts of *T. ferrooxidans* has been reported (12). Pentose phosphate isomerase was also determined spectrophotometrically by the method of Axelrod and Jange (3).

Phosphoglycerokinase, triosephosphate dehydrogenase (25), glucose-6-phosphate (G-6-P) dehydrogenase (17), and transketolase (13) were determined spectrophotometrically by current methods, as indicated. Aldolase activity was measured by the ability of extracts to form 3-phosphoglyceraldehyde (3-GAP) from fructose-1,6-diphosphate (FDP). The 3-GAP was determined by use of commercial triosephosphate dehydrogenase. Phosphogluconic acid dehydrogenase activity was determined by allowing extract to act on 6-phosphogluconic acid produced by the action of commercial G-6-P dehydrogenase on G-6-P. Production of reduced nicotinamide adenine dinucleotide phosphate (NADPH₂) before and after addition of extract was observed as an increase in OD at 340 m μ . Phosphohexoisomerase was determined by replacing the G-6-P in the G-6-P dehydrogenase reaction by fructose-6-phosphate (F-6-P). Commercial dehydrogenase was used to remove rapidly any G-6-P formed. Approximately 7% contamination of the commercial F-6-P by G-6-P necessitated prior incubation of this substrate with the G-6-P dehydrogenase before addition of extract. Once having demonstrated phosphohexoisomerase activity in the extracts, fructose diphosphatase was determined by measuring the rate of G-6-P formation by the action of extract on FDP.

Transaldolase was qualitatively determined by a procedure coupling several endogenous activities—pentose phosphate isomerase, transketolase, transaldolase, and phosphohexoisomerase. The success of this procedure depended upon the ability of extracts to produce G-6-P from R-5-P. G-6-P dehydrogenase was added to allow rapid detection of the G-6-P.

The products of CO₂ fixation were separated by paper chromatography, on 18 by 22 inch (45.7 by 55.9 cm) sheets of Whatman no. 1 papers prepared according to the method of Eggleston and Hems (11). Reaction mixtures were deproteinized after 5 to 9 hr by the addition of perchloric acid to a final concentration of 7 to 10%. The supernatant fluid was neutralized to pH 7 by addition of 5 N KOH and was allowed to stand overnight at 2 to 4 C. After centrifugation,

the supernatant fluid was lyophilized, and the residue was redissolved in one-fifth the original volume of distilled water. The concentrate was shaken with approximately 1 g of Dowex-50 cation-exchange resin and decanted, and samples were applied to the chromatograms. The solvent systems employed for separation (29) consisted of isobutyric acid — 1 N NH_4OH —0.1 M EDTA (100:60:1.6), and *n*-butanol-propionic acid-water (375:180:245). Each solvent required 16 to 20 hr for development, and the papers were air-dried after development. Known sugar phosphates were localized and identified by aniline-acid phthalate spray, bromphenol blue spray, and the acid molybdate spray of Haynes and Ischerwood (cited in 7). Radioactively labeled materials were localized by autoradiography. The developed chromatograms were placed in direct contact with 14 by 17 inch (35.8 by 43.2 cm) sheets of Dupont Cronex II X-ray film sandwiched between two 0.25 inch (0.6 cm) sheets of plywood, and placed in the dark for 2 weeks. Such extended exposure times were necessary because of the relatively low specific activity of the labeled bicarbonate employed.

RESULTS

Cell-free extracts of *T. ferrooxidans* were found to fix rapidly carbon dioxide in a system consisting of Tris buffer, MgCl_2 , labeled sodium bicarbonate, RuDP, and extract. With the crude extract preparations used in this study, R-5-P and ATP could be substituted for the RuDP, with comparable rates and amounts of fixation, whereas the use of 3-GAP, FDP, or 3-phosphoglyceric acid (3-PGA) with addition of reduced nicotinamide adenine dinucleotide (NADH_2) allowed lesser amounts and rates of fixation (Table 1). As a rule, R-5-P and RuDP were essentially quantitatively converted to labeled products, whereas lower levels of fixation were noted for the other substrates, which, like R-5-P, required ATP for fixation. A small amount of fixation was obtained with either G-6-P or F-6-P alone, but the addition of F-6-P to reaction mixtures containing 3-GAP resulted in greater fixation than with either of these compounds alone (Table 2).

The principal product of fixation with R-5-P, FDP, or 3-GAP was shown to co-chromatograph with known 3-PGA and to react in a fashion identical to that of authentic 3-PGA in specific enzyme assays (glyceraldehyde-3-phosphate dehydrogenase). In addition to this major product, a smaller amount of phosphoenolpyruvic acid (PEP) was also formed by all reaction mixtures and was demonstrated by co-chromatography. Those reaction mixtures containing FDP or 3-GAP also showed a small amount of labeled G-6-P, a product which appeared in small amounts in tests with R-5-P as substrate only when NADH_2 was added to the initial mixture.

TABLE 1. Carbon dioxide fixation by extracts of *Thiobacillus ferrooxidans* with various substrates^a

Substrate	Amt of substrate	CO ₂ fixed	Time	ATP requirement
	μmoles	μmoles	hr	
R-5-P.....	3	2.65	2	+
RuDP.....	3	2.22	2	—
FDP.....	3	0.46	3	+
F-6-P.....	4	0.06	3	
G-6-P.....	3	0.02	3	
3-GAP.....	3	0.26	5	+
3-PGA.....	3	0.09	5	
3-PGA + NADH_2^b	3	0.28	5	+
Pyruvate.....	3	0.08	1	
Ribose.....	5	0.00	5	
Xylose.....	5	0.00	5	

^a Reaction mixtures contained in a total volume of 3.0 ml: 250 μmoles of Tris chloride, pH 8.0; 5 to 10 μmoles of ATP; 20 μmoles of MgCl_2 ; 80 μmoles of $\text{NaH}^{14}\text{CO}_3$ (specific activity = 11,000 counts per min per μmole); extract (approximately 3 to 8 mg of protein); and substrate as shown.

^b Included in reaction mixture was 5 μmoles of NADH_2 .

TABLE 2. Carbon dioxide fixation by extracts of *Thiobacillus ferrooxidans* with various substrates^a

Substrate	Amt of substrate	Amt of CO ₂ fixed at	
		1 hr	9 hr
	μmoles	μmoles	μmoles
FDP + 3-GAP....	4 each	1.32	2.34
F-6-P + 3-GAP...	4 each	1.04	1.96
FDP.....	8	1.04	1.66
3-GAP.....	8	0.62	1.44
F-6-P.....	8	0.13	0.42
R-5-P.....	8	4.90 (3 hr)	

^a Conditions were as described in Table 1, with substrate as indicated above.

The ability of these extracts to utilize R-5-P as substrate for ATP-dependent CO₂ fixation, with formation of labeled 3-PGA, is ample proof of the presence of active pentose phosphate isomerase and phosphoribulokinase as well as carboxydismutase. The accumulation of a cysteine-carbazole positive substance by incubation of the extracts with R-5-P (Table 3) is a further indication of the presence of pentose phosphate isomerase.

The effect of varying ATP concentration on the rate of ATP-dependent CO₂ fixation is shown in Fig. 1. The K_m for ATP in this system was calculated to be 0.91 mM. Preliminary reports of the

TABLE 3. *Pentose phosphate isomerase activity in extracts of Thiobacillus ferrooxidans*^a

Time (min)	OD at 520 mμ ^b	
	Boiled extract control	Complete system
0	0	0.004
5	0	0.135
60	0	0.160

^a Reaction vessel contained in a 2.0-ml volume: 170 μmoles of Tris chloride, pH 7.0; 2 μmoles of R-5-P; and 0.34 mg of extract protein. Held at 30 C.

^b Samples of 0.5 ml were added to 6 ml of 25.4 N H₂SO₄, followed by 0.2 ml of 0.12% (w/v) carbazole in absolute ethyl alcohol, and 0.2 ml of 1.5% (w/v) cysteine·HCl in water. Held at 30 C for 30 min for color development.

FIG. 1. Effect of varying ATP concentration on rate of CO₂ fixation by extracts of *Thiobacillus ferrooxidans*. Each reaction vessel contained in a total of 3.0 ml: 250 μmoles of Tris chloride, pH 8.0; 5 μmoles of R-5-P; 20 μmoles of MgCl₂; 80 μmoles of NaH¹⁴CO₃ (specific activity = 11,000 counts per minute per μmole); 6 mg of extract protein; and ATP as indicated. Samples of 0.3 ml were taken at 1 hr. Velocity is expressed as counts per minute in 3.0 ml per hr.

competitive inhibition of phosphoribulokinase by adenosine-5'-monophosphate (AMP) in these extracts have appeared elsewhere (12). In addition to the effect on rate of ATP-dependent CO₂ fixation, ATP has been found to have a profound effect on the amount of fixation achieved (Table 4). Each micromole of ATP added to the reaction vessel resulted in the fixation of 1.5 μmoles of CO₂. Adenosine-5'-diphosphate (ADP) may substitute for ATP in these crude extracts, which have been shown to contain adenylate kinase

TABLE 4. *Effect of ATP and ADP on amount of CO₂ fixed by extracts of Thiobacillus ferrooxidans*^a

Nucleotide added	Amt added	Amt of CO ₂ fixed
	μmoles	μmoles
ATP	0.0	0.016
ATP	0.5	0.84
ATP	1.0	1.60
ATP	2.0	2.96
ADP	0.5	0.41
ADP	1.0	0.71
ADP	1.5	1.01
ADP	2.0	1.20
ADP	3.0	1.54

^a Reaction vessel contained in 3.0 ml: 250 μmoles of Tris chloride, pH 8.0; 5 μmoles of R-5-P; 20 μmoles of MgCl₂; 80 μmoles of NaH¹⁴CO₃ (11,000 counts per min per μmole); and extract. Those receiving ATP contained 2.5 mg of extract protein, final sample taken at 22 hr. Those receiving ADP contained 5.0 mg of extract protein, and final samples were taken at 10 hr.

(N. L. Gale, M.S. Thesis, Brigham Young Univ., Provo, Utah, 1964). Each micromole of ADP allowed the fixation of 0.5 μmole of CO₂ (Table 4). These results agree with a strict requirement for ATP in the phosphoribulokinase step and the formation of 1 mole of ATP from 2 moles of ADP through adenylate kinase involvement. The complete utilization of ATP seen here is made possible by the strongly favored formation of RuDP by the phosphoribulokinase system (J. Hurwitz, *Federation Proc.* 14:230, 1955). The extended time periods required for complete utilization of ATP are necessitated by the accumulation of AMP, which acts as a competitive inhibitor of the phosphoribulokinase of *T. ferrooxidans*. Nevertheless, the reaction apparently goes to completion, and chromatograms demonstrate that, in these reaction mixtures, ATP and ADP are essentially quantitatively converted to AMP.

The effects of varying the concentrations of RuDP and bicarbonate on the activity of carboxydismutase are shown in Fig. 2 and 3, respectively. The values of *K_m* for RuDP (1.15 × 10⁻⁴ M) and bicarbonate (5 × 10⁻² M) are in good agreement with values reported from photosynthetic organisms (21, 32).

Tests for various enzyme activities. The results of the analyses of products of CO₂ fixation as described above were suggestive of a functional Calvin cycle in extracts of *T. ferrooxidans*, although the accumulation of relatively large amount of 3-PGA, even in the presence of added NADH₂, raised the possibility that one or more

FIG. 2. Effect of varying concentration of RuDP on rate of CO_2 fixation by extracts of *Thiobacillus ferrooxidans*. Reaction mixtures of 3.0 ml volume contained: 250 μmoles of Tris chloride; pH 8.0; 20 μmoles of MgCl_2 ; 80 μmoles of $\text{NaH}^{14}\text{CO}_3$ (specific activity = 12,600 counts per min per μmole); 1.5 mg of extract protein; and RuDP as shown. Velocity is expressed as counts per minute in 3.0 ml per 30 min.

FIG. 3. Effect of varying bicarbonate concentration on rate of CO_2 fixation by extracts of *Thiobacillus ferrooxidans*. Reaction mixtures contained in 3.0 ml; 250 μmoles of Tris chloride, pH 8.0; 5 μmoles of ATP; 3 μmoles of R-5-P; 20 μmoles of MgCl_2 ; 10 mg of extract protein; and $\text{NaH}^{14}\text{CO}_3$ (11,000 counts per min per μmole). Velocity is expressed in terms of micromoles of CO_2 fixed in 3.0 ml per 30 min.

of the enzymes involved in this cycle may be absent or reduced in activity in the extract preparations. Therefore, a study was conducted to determine whether the various enzymes of the Calvin or the associated hexose monophosphate pathway could be detected in these cell-free preparations, and whether the relative activities of these en-

zymes might account for the accumulation of labeled products as described above. The results of this study are summarized in Table 5. The activity of triose-phosphate dehydrogenase was markedly enhanced by the presence of GSH, as was the case with the similar enzyme from *T. thiooxidans* (25), and the reported activity shown in Table 5 is based upon experiments in which this reducing compound was included in the mixture. However, the addition of GSH to crude systems utilizing R-5-P was found to cause serious inhibition of CO_2 fixation, and for this reason was generally omitted from reaction mixtures involving the incorporation of labeled CO_2 . The accumulation of 3-PGA as the principal product of CO_2 fixation in these cell-free systems was, therefore, probably due at least in part to the restricted activity of this critical enzyme.

The assay for two of the enzymes of the hexose monophosphate pathway is shown in detail in Fig. 4. Although ATP was not required for 6-phosphogluconic acid dehydrogenase activity, its presence did appear to enhance the rate of reaction, probably because of removal of the product ribulose-5-phosphate in the strongly favored formation of RuDP. At pH 8.0, normally employed for CO_2 fixation studies and optimum for the G-6-P dehydrogenase enzyme, the activity of the 6-phosphogluconate dehydrogenase was markedly less than at pH 7.0.

Transaldolase and the production of G-6-P from R-5-P. The ability of extracts to produce G-6-P from R-5-P, which involves the action of transaldolase and several other endogenous enzymes, is shown in Fig. 5. In the presence of added ATP, very little G-6-P was found to accumulate. Equilibrium obviously favors the formation of RuDP in the presence of ATP (J. Hurwitz, Federation Proc.).

DISCUSSION

The demonstrated presence of the enzymes of the Calvin cycle in cell-free extracts of *T. ferrooxidans* suggests this pathway is operative in whole cells of this microorganism. Although rates of reaction are reported for the various enzymes in the text of this work, these figures are intended only to demonstrate a detectable level of activity and do not necessarily reflect the true activities within the living cell. No attempt was made to ascertain optimal conditions for each enzyme studied, and assay conditions were generally selected on the basis of literature reports on similar enzymes from other sources. The reported rates do, however, serve to provide possible reasons for the appearance of certain compounds among the products of CO_2 fixation by cell-free extracts

TABLE 5. Enzymes of Calvin cycle and hexose monophosphate pathway in extracts of *Thiobacillus ferrooxidans*

Enzyme	Activity ^a	Mixture (3 ml) ^b
Triose phosphate dehydrogenase and phosphoglycerokinase.....	0.33, NADH ₂ oxidized	A
Aldolase.....	0.25, FDP cleaved	B
G-6-P dehydrogenase (pH 8.0).....	0.38, NADPH ₂ formed	C
6-Phosphogluconate dehydrogenase (pH 7).....	0.35, NADPH ₂ formed	C
Phosphohexoisomerase.....	0.48, G-6-P formed	D
Fructose diphosphatase.....	0.48, G-6-P formed	E
Transketolase.....	0.47, 3-GAP formed	F

^a Expressed in micromoles of indicated compound per hour per milligram of protein.

^b Mixture A = 250 μ moles of Tris chloride, pH 8.0; 2 μ moles of ATP; 2 μ moles of 3-PGA; 30 μ moles of GSH; 0.5 μ moles of NADH₂; and 3.3 mg of extract protein (NADPH₂ could not substitute). B = 200 μ moles of Tris chloride, pH 8.0; 4 μ moles of FDP; 3 μ moles of NAD⁺; 50 μ moles of sodium phosphate buffer, pH 8.0; 20 μ moles of CoCl₂, 0.2 mg of triosephosphate dehydrogenase (Sigma Chemical Co.); and 6 mg of extract protein. C, see Fig. 4. D = 150 μ moles of Tris chloride, pH 8.0; 20 μ moles of MgCl₂; 2 μ moles of NADP⁺; 0.03 ml of G-6-P dehydrogenase; 2 μ moles of F-6-P. When no further change in OD was apparent, 3.4 mg of extract protein was added. E = same as D, except for replacement of F-6-P by 2 μ moles of FDP. F = 100 μ moles of Tris chloride, pH 8.0; 3 μ moles of R-5-P; 0.5 μ mole of NADH₂; 0.1 ml of mixed suspension of triose isomerase and α -glycerophosphate dehydrogenase (Sigma Chemical Co.); and 3.4 mg of extract protein.

FIG. 4. Glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase in extracts of *Thiobacillus ferrooxidans*. Reacting mixtures contained in 3.0 ml: 150 μ moles of Tris chloride, pH 8.0; 10 to 20 μ moles of MgCl₂; and the following additions: ●, 1 μ mole of NADP⁺, 2 μ moles of G-6-P, 3.4 mg of extract protein; ▲, 1 μ mole of NADP⁺, 0.03 ml of Sigma Type IV G-6-P dehydrogenase, 3.0 mg of extract protein; ○, 0.7 μ mole of G-6-P, 2 μ moles of NADP⁺, 0.03 ml of Sigma Type IV G-6-P dehydrogenase, and 1 μ mole of ATP; △, same as ○, except 200 μ moles of Tris chloride at pH 7.0 rather than pH 8.0 were used. Star indicates 6 mg of extract protein added.

FIG. 5. Glucose-6-phosphate formation from ribose-5-phosphate by extracts of *Thiobacillus ferrooxidans*. Reaction mixtures contained in 3.0 ml volume: 100 μ moles of Tris chloride, pH 8.0; 5 μ moles of R-5-P; 10.2 mg of extract protein; 0.05 ml of Sigma Type IV G-6-P dehydrogenase; and additions as follows: △, 2 μ moles of NADP⁺ (time shown for △ only is one-fifth actual time); ○, 2 μ moles of NADP⁺ added after 2 hr; ■, 10 μ moles of ATP added at beginning, with 2 μ moles of NADP⁺ added after 2 hr. Optical density measurements were made at time of addition of NADP⁺ to reaction vessels.

as determined in the laboratory, as well as the varying amounts of fixation obtained with different substrates. The assessed rates are, of course, subject to considerable error introduced by possible side reactions that interfere with the particular enzymatic activity under examination. Furthermore, the process of extract preparation and storage may have inactivated some of the enzymes or reduced their activity from that normally found within the cell.

It would be difficult, indeed, to arrive at a single set of conditions in which each enzyme of the entire cycle would be operating optimally. Therefore, although numerous attempts have been made to obtain cyclic regeneration of RuDP for the fixation of CO_2 in cell-free systems to which only NADH_2 , ATP, and RuDP are added, consistent evidence for such regeneration is lacking. R-5-P and RuDP were quantitatively converted to labeled products, primarily 3-PGA. The accumulation of 3-PGA was probably the result of low specific activity of triosephosphate dehydrogenase in the extracts. Some slight activity of this enzyme was evident, since 3-PGA itself could serve as a substrate for significant amounts of fixation, provided that NADH_2 was also added to the reaction mixture. The fixed CO_2 observed with 3-PGA in the absence of NADH_2 was possibly due to conversion to PEP and subsequent carboxylation of this compound. No attempt was made, however, to find these four-carbon compounds. Greater amounts of fixation were noted with the use of intermediate compounds beyond the reductive step as substrates. FDP and 3-GAP served as good substrates for ATP-dependent CO_2 fixation, especially when used in combination. The use of either or both of these compounds in the absence of added NADH_2 resulted in the appearance of labeled G-6-P, a compound which appeared in reaction mixtures utilizing R-5-P as substrate only when NADH_2 was also added. The G-6-P, in any case, represented only a small fraction of the total fixed CO_2 .

It is conceivable that, although most of the pentose phosphate required for CO_2 fixation arose from FDP and 3-GAP through the transketolase and transaldolase pathway, some Ru-5-P may have been produced through the conversion of FDP to G-6-P and subsequent decarboxylation through the hexose monophosphate shunt. The result of this would be the production of NADPH_2 from small amounts of nicotinamide adenine dinucleotide phosphate (NADP^+) present in the extract. This, in turn, may have caused the reduction of nicotinamide adenine dinucleotide (NAD^+) through a transhydrogenase reaction, to provide the necessary reducing power

required by the triosephosphate dehydrogenase reaction. The end result of this would be the formation of labeled G-6-P from labeled 3-PGA. Such a scheme could account for the appearance of the labeled G-6-P in the absence of added NADH_2 in those reaction mixtures containing FDP or 3-GAP. Although these extracts can effect the formation of G-6-P from R-5-P, as long as excess ATP is not present, the G-6-P produced through transketolase and transaldolase would not be labeled. In the presence of ATP, R-5-P is quantitatively converted to RuDP, leaving no available avenue for the production of NADH_2 . Any reduction of 3-PGA which arises from the fixation of CO_2 utilizing R-5-P or RuDP must come at the expense of added NADH_2 . Thus, labeled G-6-P would be expected to appear in reaction mixtures containing R-5-P or RuDP only when NADH_2 is also added.

No specific attempt was made to measure the activity of pentose phosphate epimerase, considered to be essential to normal functioning of both the Calvin and hexose monophosphate pathways, and responsible for the interconversion of Ru-5-P and xylulose-5-phosphate. The latter compound normally arises through those reactions catalyzed by transketolase during the production of pentoses from hexoses. Although it may also be an intermediate in the formation of hexose-phosphate from R-5-P by extracts of *T. ferrooxidans*, as demonstrated in this study, its production is not obligatory, since a number of ketoses, including Ru-5-P, may serve as donor of the active glycolaldehyde in the transketolase catalyzed reaction (22).

ACKNOWLEDGMENTS

We wish to express gratitude to the National Science Foundation for its support in the form of a research grant (GB 2627) and a predoctoral fellowship (to N. L. G.) during the course of this work. Contribution by the National Cancer Institute in the form of a Special Fellowship (to J. V. B.) is also acknowledged.

Appreciation is also expressed to James Meldrum and Robert Black for their technical assistance in the laboratory.

LITERATURE CITED

1. ALEEM, M. I. H. 1965. Path of carbon and assimilatory power in chemosynthetic bacteria. I. *Nitrobacter agilis*. Biochem. Biophys. Acta 107:14-28.
2. ALEEM, M. I. H., AND E. HUANG. 1965. Carbon dioxide fixation and carboxydismutase in *Thiobacillus novellus*. Biochem. Biophys. Res. Commun. 20:515-520.
3. AXELROD, B., AND R. JANGE. 1954. Purification and properties of phosphoriboisomerase from alfalfa. J. Biol. Chem. 209:847-855.

4. BASSHAM, J. A., AND M. CALVIN. 1957. The path of carbon in photosynthesis. Prentice-Hall, Inc., Englewood Cliffs, N.J.
5. BECK, J. V. 1960. A ferrous-ion oxidizing bacterium. I. Isolation and some general physiological characteristics. *J. Bacteriol.* 79:502-509.
6. BECK, J. V., AND F. M. SHAFIA. 1964. Effect of phosphate ion and 2,4-dinitrophenol on the activity of intact cells of *Thiobacillus ferrooxidans*. *J. Bacteriol.* 88:850-857.
7. BLOCK, R. J., E. L. DURRUM, AND C. ZWEIG. 1955. A manual of paper chromatography and paper electrophoresis. Academic Press, Inc., New York.
8. BRYNER, L. C., J. V. BECK, D. B. DAVIS, AND D. G. WILSON. 1954. Microorganisms in leaching sulfide minerals. *Ind. Eng. Chem.* 46:2587-2592.
9. BRYNER, L. C., AND A. K. JAMESON. 1958. Microorganisms in leaching sulfide minerals. *Appl. Microbiol.* 6:281-287.
10. COLMER, A. R., K. L. TEMPLE, AND M. E. HINKLE. 1950. An iron-oxidizing bacterium from the acid drainage of some bituminous coal mines. *J. Bacteriol.* 59:317-328.
11. EGGLESTON, L. V., AND R. HEMS. 1952. Separation of adenosine phosphates by paper chromatography and the equilibrium constant of the myokinase system. *Biochem. J.* 52:156-160.
12. GALE, N. L., AND J. V. BECK. 1966. Competitive inhibition of phosphoribulokinase by AMP. *Biochem. Biophys. Res. Commun.* 24:792-796.
13. HORECKER, B. L., P. Z. SMYRNIOTES, AND H. KLENOW. 1953. The formation of sedoheptulose phosphate from pentose phosphate. *J. Biol. Chem.* 205:661-682.
14. HUTCHINSON, M., K. I. JOHNSTONE, AND D. WHITE. 1966. Taxonomy of acidophilic *Thiobacilli*. *J. Gen. Microbiol.* 44:373-381.
15. IVANOV, V. I., AND N. N. LYALIKOVA. 1962. Taxonomy of iron-oxidizing *Thiobacilli*. *Microbiology (USSR) (English Transl.)* 31:382-383.
16. KINSEL, N. A. 1960. New sulfur oxidizing iron bacterium: *Ferrobacillus sulfooxidans* sp. n. *J. Bacteriol.* 80:628-632.
17. KORNBERG, A. 1950. Enzymatic synthesis of triphosphopyridine nucleotide. *J. Biol. Chem.* 182:805-813.
18. LEATHEN, W. W., N. A. KINSEL, AND S. A. BRALEY, JR. 1956. *Ferrobacillus ferrooxidans*: a chemosynthetic autotrophic bacterium. *J. Bacteriol.* 72:700-704.
19. MACIAG, W. J., AND D. G. LUNDGREN. 1964. Carbon dioxide fixation in the chemoautotroph, *Ferrobacillus ferrooxidans*. *Biochem. Biophys. Res. Commun.* 17:603-607.
20. MILHAUD, G., J. P. AUBERT, AND J. MILLET. 1956. Métabolisme du carbone dans la chimioautotrophie. Cycle d'assimilation de l'anhydride carbonique. *Compt. Rend.* 243:102-105.
21. PAULSEN, J. N., AND M. D. LANE. 1966. Spinach ribulose diphosphate carboxylase. I. Purification and properties of the enzyme. *Biochemistry* 5:2350-2357.
22. RACKER, E., G. DE LA HABA, AND I. G. LEDER. 1954. Transketolase-catalyzed utilization of fructose-6-phosphate and its significance in a glucose-6-phosphate oxidation cycle. *Arch. Biochem. Biophys.* 48:238-240.
23. ROTMAN, B. 1956. On the mechanism of sonic lysis of bacteria. *J. Bacteriol.* 72:827-830.
24. SANTER, M., AND W. VISHNIAC. 1955. CO₂ incorporation by extracts of *Thiobacillus thio-parus*. *Biochim. Biophys. Acta* 18:157-158.
25. SUZUKI, I., AND C. H. WERKMAN. 1958. Chemoautotrophic carbon dioxide fixation by extracts of *Thiobacillus thiooxidans*. II. Formation of phosphoglyceric acid. *Arch. Biochem. Biophys.* 77:112-123.
26. TEMPLE, K. L., AND A. R. COLMER. 1951. The autotrophic oxidation of iron by a new bacterium: *Thiobacillus ferrooxidans*. *J. Bacteriol.* 62:605-611.
27. TRUDINGER, P. A. 1955. Phosphoglycerate formation from pentose phosphate by extracts of *Thiobacillus denitrificans*. *Biochim. Biophys. Acta* 18:581-582.
28. TRUDINGER, P. A. 1956. Fixation of carbon dioxide by extracts of the strict autotroph *Thiobacillus denitrificans*. *Biochem. J.* 64:274-286.
29. TYSZKIEWICZ, E. 1962. An improved solvent system for the paper chromatography of phosphate esters. *Anal. Biochem.* 3:164-172.
30. VISHNIAC, W., AND P. A. TRUDINGER. 1962. Symposium on autotrophy. V. Carbon dioxide fixation and substrate oxidation in the chemosynthetic sulfur and hydrogen bacteria. *Bacteriol. Rev.* 26:168-175.
31. WARBURG, O., AND W. CHRISTIAN. 1941. Isolierung und Kristallisation des Gärungsferments Enolase. *Biochem. Z.* 310:384-412.
32. WEISSBACH, A., B. L. HORECKER, AND J. HURWITZ. 1956. The enzymatic formation of phosphoglyceric acid from ribulose diphosphate and CO₂. *J. Biol. Chem.* 218:795-810.