Coupling of Phosphorylation and Carbon Dioxide Fixation in Extracts of *Thiobacillus thioparus*[1]

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ABSTRACT

JOHNSON, EMMETT J. (University of Mississippi Medical Center, Jackson), AND HARRY D. PECK, JR. Coupling of phosphorylation and carbon dioxide fixation in extracts of *Thiobacillus thioparus*. J. Bacteriol. **89**:1041–1050. 1965.—A cell-free system from *Thiobacillus thioparus* which fixes large quantities of $C^{14}O_2$ in the presence of ribose-5-phosphate, adenosine triphosphate (ATP), and Mg^{++} has been described. The specific activity (0.041 μmole of ribulose-1,5-diphosphate min^{-1} mg^{-1} protein) of the CO_2-fixing system approaches that of green plants, and is further evidence for the importance of the role of carboxydismutase in the thiobacilli. In addition to ATP, adenosine diphosphate (ADP) and other nucleoside triphosphates served with varying degrees of effectiveness for the fixation of $C^{14}O_2$. The ATP requirement for CO_2 fixation was partially replaced under aerobic conditions by a combination of SO_3^{-}, $PO_4^{=}$, and adenosine monophosphate (AMP). Phosphorylation and CO_2 fixation were separated in time by first incubating SO_3^{-} and AMP aerobically, and then anaerobically introducing $C^{14}O_3^{-}$ and ribose-5-phosphate into the reaction mixture. During the first incubation, $P^{32}O_4^{=}$ was esterified into nucleotides, mainly ADP, and in the second incubation $C^{14}O_2$ was fixed, with the concomitant utilization of almost equal amounts of the esterified phosphate. These data provide the first in vitro evidence for the mechanism of the coupling of CO_2 fixation and phosphorylation in *T. thioparus*. The fixation of $C^{14}O_2$ was shown to be almost completely inhibited by AMP. This inhibition was not due to the conversion of ATP to ADP by adenylic kinase, or to the binding of magnesium by the nucleotide. The inhibition was specific for AMP, since other mononucleotides, adenosine, and adenine did not inhibit. The AMP regulation of CO_2 fixation may represent a basic control mechanism in autotrophic metabolism.

Chemosynthetic autotrophs exhibit a mode of life unique among living organisms, deriving their total energy for growth from the oxidation of inorganic substrates and their total carbon from the fixation of CO_2. The earliest attempts to explain the relationship between energy production and CO_2 fixation in chemosynthetic autotrophic bacteria are found in the postulations of Vogler and Umbreit (1942) concerning the separation in time of phosphorylation and CO_2 fixation. They observed that, in the absence of CO_2 fixation, whole cells of *Thiobacillus thiooxidans* can oxidize available substrates and store

the energy in a form that subsequently, in the absence of oxidizable substrates, can be utilized to fix CO_2. Objections were raised by Baalsrud and Baalsrud (1952), because examination of the data quantitatively revealed an irreconcilable divergence from expectation on the basis of theoretical considerations concerning the amount of CO_2 fixed and of orthophosphate (P_i) utilized. The proposals of Umbreit (1960) seem to be generally correct, although perhaps not warranted by the data existing at the time; however, evidence from experiments that include all the critical variables is still lacking (Larsen, 1960).

Vogler, LePage, and Umbreit (1942) suggested the existence of oxidative phosphorylation, because they were able to show that, at low concentrations, 2,4-dinitrophenol stimulated the respiration of *T. thiooxidans*. As yet, however, there is no direct evidence for oxidative phosphorylation in these organisms, and the exact relationship between energy production and CO_2 fixation is still largely a matter of conjecture.

Indirect evidence for oxidative phosphorylation in thiobacilli has been reviewed by Vishniac and Trudinger (1962); however, oxidative phosphorylation has not as yet been demonstrated to occur in cell-free preparations of any thiobacilli. At present, only a substrate phosphorylation insensitive to 2,4-dinitrophenol has been demonstrated in thiobacilli. This substrate-level phosphorylation occurs during the oxidation of sulfite to sulfate, and it might be employed to demonstrate the in vitro coupling of phosphorylation with CO_2 fixation (Peck, 1962). This pathway of sulfite oxidation involves the enzyme, adenosine-5-phosphosulfate (APS) reductase, which, in addition to reducing APS, brings about the oxidation of sulfite in the presence of adenosine monophosphate (AMP), with the formation of APS (Peck, 1961):
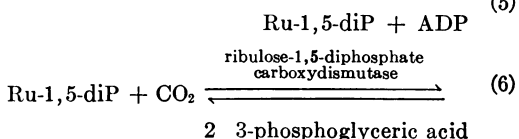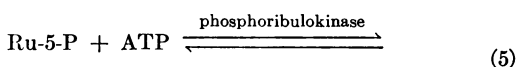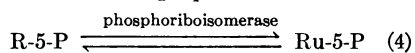
$$SO_3^- + AMP \xrightleftharpoons{APS\text{-}reductase} APS + 2e \quad (1)$$
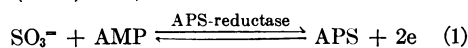
The high-energy sulfate thus formed participates in a reaction with P_i, catalyzed by the enzyme, adenosine diphosphate (ADP)-sulfurylase, and results in the production of ADP and sulfate:

$$APS + Pi \xrightleftharpoons{ADP\text{-}sulfurylase} ADP + SO_4^- \quad (2)$$
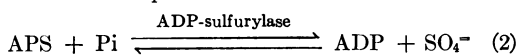
Adenylic kinase, which is also present in extracts of these organisms, will convert ADP to adenosine triphosphate (ATP):

$$2\ ADP \xrightleftharpoons{adenylic\ kinase} AMP + ATP \quad (3)$$
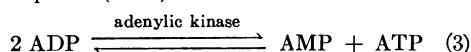
The requisite enzymes for the fixation of CO_2 with ribulose-1,5-diphosphate (Ru-1,5-diP) have also been demonstrated in several species of thiobacilli (Santer and Vishniac, 1955; Trudinger, 1956; Suzuki and Werkman, 1958; Iwatsuka, Kuno, and Maruyama, 1962). The enzymes catalyzing the fixation of CO_2 appear to be similar to those described in green plants (Elsden, 1962); beginning with ribose-5-phosphate (R-5-P), fixation involves the enzymes phosphoriboisomerase, phosphoribulokinase, and Ru-1,5-P carboxydismutase (equations 4, 5, and 6). In order that CO_2 fixation be dependent upon phosphorylation, it is necessary to begin with R-5-P or ribulose-5-phosphate (Ru-5-P) and CO_2 as shown in the following equations:

$$R\text{-}5\text{-}P \xrightleftharpoons{phosphoriboisomerase} Ru\text{-}5\text{-}P \quad (4)$$

$$Ru\text{-}5\text{-}P + ATP \xrightleftharpoons{phosphoribulokinase}$$
$$Ru\text{-}1,5\text{-}diP + ADP \quad (5)$$

$$Ru\text{-}1,5\text{-}diP + CO_2 \xrightleftharpoons[carboxydismutase]{ribulose\text{-}1,5\text{-}diphosphate}$$
$$2\ \ 3\text{-}phosphoglyceric\ acid \quad (6)$$

As suggested previously (Peck, 1962), the requisite enzymes for oxidation of sulfite coupled to the production of high-energy phosphate, as well as for the fixation of CO_2 (see above), have been demonstrated in extracts of these organisms. Thus, it should now be possible to reproduce the experiments involving simultaneous energy production and CO_2 fixation and sequential energy production and CO_2 fixation in a cell-free system. The present communication describes the properties of a CO_2-fixing system from *T. thioparus* and the sequential coupling of phosphorylation and CO_2 fixation in a cell-free extract of this organism. In addition, a possible mechanism for the regulation of CO_2 fixation is presented.

MATERIALS AND METHODS

Cultural procedures and preparation of extracts. *T. thioparus* (ATCC 8158) was grown in 40 liters of the inorganic medium described by Santer, Boyer, and Santer (1959), with the following modifications: $Na_2S_2O_3 \cdot 5H_2O$, 8.0 g; NH_4Cl, 0.5 g. The thiosulfate and phosphates were autoclaved separately and added aseptically just prior to inoculation. Cells were grown at 30 C with forced aeration for 5 to 6 days, and the pH was adjusted when required by addition of a 10% solution of Na_2CO_3. After harvesting the cells in a refrigerated Sharples centrifuge, a 25 to 50% suspension of the cell paste was made in 0.33 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 7.1 to 7.5; this suspension was passed once, or occasionally twice, through a French pressure cell (American Instrument Co., Inc., Silver Spring, Md.). If the extract was too viscous, 20 μg of deoxyribonuclease were added, and the extract was allowed to stand 5 to 10 min at 30 C. Later, it was observed that greater retention of activity is achieved if the extract is held in an ice bath until the gel-like material liquifies, as indicated by visual inspection (usually several hours, but it can be left overnight). The extract was then centrifuged at 20,000 × g for 30 min. Where indicated, the clarified extract was chromatographed on a coarse Sephadex G-25 column, 15 mm × 300 mm (16-ml capacity). In some cases, the Sephadex-treated extracts were aged approximately 24 hr at 2 to 4 C to reduce endogenous CO_2 fixation.

Respiration and CO_2 fixation. Oxygen uptake was measured with Warburg vessels (20 ml) at 30 C (gas phase, air) without NaOH in the center well. Double side arm flasks were used; one side arm contained Na_2SO_3 and $MgCl_2$, and the other contained R-5-P and $Na_2C^{14}O_3$. One side arm was sealed with a vented plug to change the gas phase when required. The second side arm was sealed with a serum cap to allow additions of perchloric acid to stop the reaction and NaOH to absorb the unfixed CO_2. The flasks were equilibrated 5 min

before adding the substrate, and oxidation was allowed to proceed for 10 min in air. In those experiments in which oxidation was separated from CO_2 fixation, the flasks were flushed with helium for 5 min after the oxidation period, and then the R-5-P and $Na_2C^{14}O_3$ were tipped from one sidearm for the fixation of CO_2. After 10 min, 0.3 ml of 10% perchloric acid was injected into the side arm through the serum cap by use of a tuberculin syringe with a 21-gauge needle. The perchloric acid was then added to the main compartment to stop the reaction and to release the unfixed $C^{14}O_2$. Subsequently, 0.3 ml of 10% NaOH was injected into the side arm, and the flasks were shaken for 10 min at 30 C so that the unfixed $C^{14}O_2$ would be absorbed. Radioactivity retained in the deproteinized reaction mixtures was employed as a measure of $C^{14}O_2$ fixed.

Phosphate measurements. In experiments in which changes in nucleotide phosphates are reported, P^{32}-labeled orthophosphate was included in the reaction mixtures to facilitate analyses. Nucleotides were adsorbed onto charcoal from protein-free reaction mixtures. After washing the charcoal four times with 0.05 M acetate buffer (*p*H 5.0) and once with distilled water, the labile phosphate was hydrolyzed (1 N HCl for 15 min at 100 C). Radioactivity of the hydrolysate was determined by means of a Packard liquid scintillation spectrometer.

Chromatography and electrophoresis. The protein-free reaction mixtures were neutralized with 30% KOH, and the $KClO_4$ was removed by centrifugation. The samples were then frozen and thawed several times, and any further precipitate was similarly removed. The reaction mixtures were then extracted with 5 ml of petroleum ether to remove volatile acids, evaporated to dryness, and brought back into solution with 0.2 to 0.3 ml of glass-distilled water. The sugar phosphates were chromatographed on Whatman no. 1 filter-paper strips or sheets with descending development at 20 to 25 C. The solvent systems used were isopropyl ether–formic acid (90%, w/v; 3:2) and 80% ethanol-0.8% acetic acid. The former system was run for 6 to 8 hr and the latter for 12 to 13 hr. The chromatograms were sprayed with a mixture of perchloric acid, HCl, and ammonium molybdate (Hanes and Isherwood, 1949), and the sugar phosphates were located with ultraviolet light. Electrophoresis of nucleotides was carried out as described by Robbins and Lipmann (1958), by use of 0.03 M citrate buffer (*p*H 5.5). For electrophoresis, nucleotides were adsorbed on acid-washed charcoal (100 mg per 5 ml), which was then washed five times with glass-distilled water before eluting the nucleotides with two 3-ml portions of ammoniacal ethanol. The eluates were combined, evaporated to dryness, dissolved in 0.2 ml of glass-distilled water, and subjected to high-voltage paper electrophoresis. Nucleotides were also chromatographed on diethylaminoethyl (DEAE)-cellulose paper sheets at 20 to 25 C and on Whatman no. 1 filter-paper sheets at 20 to 25 C in iso-

butyric acid-NH_3-water (577:38:385; Jacobson, 1964).

Radioactivity measurements. Radioactivity was measured in a liquid scintillation spectrometer (Packard Instrument Co., LaGrange, Ill.) in 10 ml of the following solvent: 2,5-diphenyloxazole, 4 g; 1,4-bis-2'(5'-phenyloxazolyl) benzene, 100 mg; toluene, 700 ml; absolute ethanol, 300 ml. $P^{32}O_4$ and $BaC^{14}O_3$ were purchased from the Oak Ridge National Laboratory, and $BaC^{14}O_3$ was converted to $Na_2C^{14}O_3$.

RESULTS

Requirements for CO_2 fixation. Before testing the ability of extracts from *T. thioparus* to couple phosphorylation with CO_2 fixation, it was necessary to demonstrate that the extracts had the capacity for fixing reasonable amounts of CO_2, beginning at some point in the pathway of CO_2 fixation that would include at least one energy-requiring step. The work of Santer and Vishniac (1955) showed that small amounts of CO_2 (2.5 mμmoles) were fixed by extracts of *T. thioparus* with Ru-1,5-diP, but not with R-5-P, as the acceptor. However, Trudinger (1956) was able to demonstrate the fixation of $C^{14}O_2$ with R-5-P in extracts of *T. denitrificans*, and similar results were obtained with extracts of *T. thiooxidans* (Suzuki and Werkman, 1958; Iwatsuka et al., 1962). Beginning with R-5-P, the system should necessarily be ATP-dependent (equations 5 and 6). Table 1 shows that our extract from *T. thioparus* fixes relatively large amounts of $C^{14}O_2$ (0.041 μmole of Ru-1,4-diP min^{-1}mg^{-1} protein) in the overall system. This compares with a specific activity of 0.08 to 0.09 for Ru-1,5-diP carboxydismutase in the soluble extract of spinach leaves (Racker, 1962). If phosphoribulokinase was rate-limiting in our extracts of *T. thioparus*, the specific activity of the carboxylase reported here would be a minimal rather than a maximal value. The system is completely dependent on Mg^{++} and has a strict requirement for ATP and R-5-P. Ethylenediaminetetracetic acid (EDTA) and NaF were initially added to reaction mixtures because they are required for maximal activity in the phosphorylating system (Peck and Fisher, 1962). Omission of EDTA did not appreciably affect the fixation, probably because Mg^{++} was in excess. However, omission of NaF resulted in an increase in fixation, and it was concluded that NaF inhibits this system. The addition of fructose-1,6-diphosphate, glucose-6-phosphate, and 3-phosphoglyceric acid (3-PGA) to the system resulted in a decrease in the amount of $C^{14}O_2$ fixed. The first two compounds probably shunted R-5-P into pathways of utilization other than CO_2 fixation, thus decreasing the amount of R-5-P available for the fixation process.

TABLE 1. *Requirements for CO_2 fixation in extracts of Thiobacillus thioparus*

System	$C^{14}O_2$ fixed
	μmoles
Complete*	2.73
Minus Mg	0.00
Minus ATP	0.02
Minus R-5-P	0.03
Minus EDTA	2.62
Minus NaF	3.52
Plus fructose-1,6-diphosphate	2.01
Plus glucose-6-phosphate	2.20
Plus 3-phosphoglyceric acid	1.82
Minus R-5-P + fructose-1,6-diphosphate	0.12
Minus R-5-P + glucose-6-phosphate	0.03
Minus R-5-P + 3-phosphoglyceric acid	0.02

* The complete system contained in μmoles: Tris (pH 8.0), 330; EDTA, 10; NaF, 10; $MgCl_2$, 30; ATP, 10; R-5-P, 10; $Na_2C^{14}O_3$, 5 (10^6 counts per min per μmole); 0.5 ml of a Sephadex-treated extract (8.5 mg of protein). The following substances were added in the amount of 10 μmoles: fructose-1,6-diphosphate, glucose-6-phosphate, and 3-phosphoglyceric acid. The total volume of the reaction mixture was 2.7 ml. The incubation period was 10 min at 30 C under helium, and the reaction was stopped with 0.3 ml of 10% perchloric acid.

The reduction of fixation effected by 3-PGA may be due to product inhibition. None of these compounds could replace R-5-P in the fixation system, as no significant amount of $C^{14}O_2$ was fixed in its absence.

Chromatography and radioautography of the neutralized aqueous phase of ether-extracted protein-free reaction mixtures revealed only two radioactive spots corresponding to R-5-P and fructose-1,6-diphosphate. Short pulses with labeled carbonate were not done; therefore, it might not be expected that the label would be found in 3-PGA, as 3-PGA is rapidly converted into other intermediates of hexose synthesis. The compounds found in this system are known intermediates of the pathway of hexose synthesis from CO_2 (Bassham et al., 1954), and it is known from the work of others (Trudinger, 1956; Suzuki and Werkman, 1958) that extracts of thiobacilli contain many of the enzymes involved in hexose metabolism.

Since the CO_2-fixing system was being employed essentially to assay for the formation of high-energy phosphate, it was of interest to determine the nucleotide specificity of the reaction in these extracts. Although Hurwitz et al. (1956) indicated that the purified phosphoribulokinase from spinach is highly specific for ATP

(Table 2), all of the nucleotides tested supported CO_2 fixation. In addition, the activity of these nucleotides was approximately the same as ATP, except for cytidine triphosphate (CTP). ADP, the product of the substrate phosphorylation, also stimulated CO_2 fixation, although not as effectively as ATP. Whether the utilization of a variety of nucleotides is an indication of the nucleotide nonspecificity of this phosphoribulokinase or a reflection of nucleotide kinases is not known and must await purification of the enzyme. Nevertheless, this result did indicate that any high-energy phosphate could be utilized for the fixation of CO_2.

Properties of CO_2-fixing enzymes. Before testing the ability of the extract to couple sulfite oxidation and ATP formation to CO_2 fixation, it was necessary to determine the experimental response of the CO_2-fixing enzymes to a range of concentrations of ATP, because the amount of ATP produced from sulfite oxidation in these extracts would probably be small. The activity was proportional to the concentration of ATP up to about 5 μmoles of ATP, at which point the system appeared to be saturated with respect to ATP (Fig. 1). This response of the CO_2-fixing system indicated that it could be utilized essentially as an assay for ATP. A study of the amount of CO_2 fixed as a function of time (Fig. 2) showed that the activity was linear for the duration of the experiment. Consequently, a 10-min incubation period for CO_2 fixation was selected. The relationship between CO_2 fixation and ATP utilization is less than stoichiometric and probably results from the hydrolysis of the added ATP, since NaF was omitted from this assay system. With NaF and lower concentrations of ATP, there is a 1:1

TABLE 2. *Nucleotide specificity of CO_2 fixation in extracts of Thiobacillus thioparus**

Additions†	$C^{14}O_2$ fixed
	μmoles
None	0.02
ATP	2.62
ITP	2.42
GTP	2.15
CTP	1.18
UTP	2.49
dATP	2.37

* The system was the same as in Table 1, except that 10 μmoles of each nucleotide were added; 0.5 ml of a Sephadex-treated extract was the source of enzyme (9.2 mg of protein).

† Symbols: ITP, inosine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate.
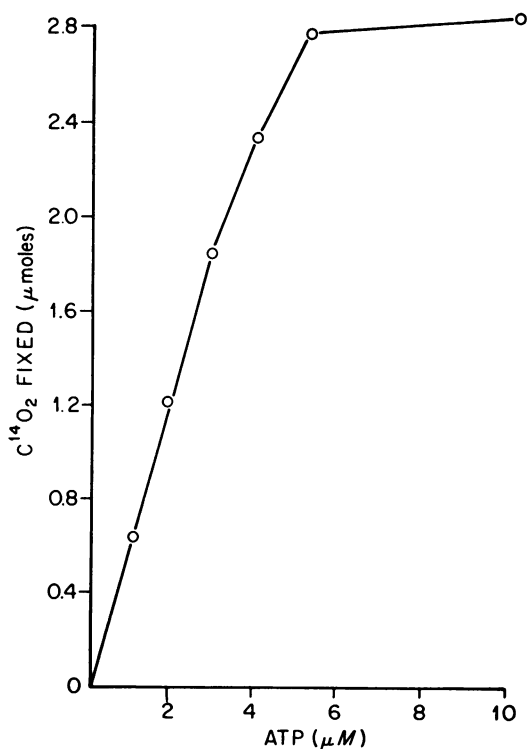
FIG. 1. *Relationship between ATP concentration and $C^{14}O_2$ fixation in a cell-free system from Thiobacillus thioparus. The complete system was the same as in Table 1, except that fluoride was omitted; 0.5 ml of a Sephadex-treated extract was the enzyme source (6.6 mg of protein).*

FIG. 2. *Relationship between incubation time and $C^{14}O_2$ fixation in a cell-free system from Thiobacillus thioparus. The complete system was the same as in Table 1, except that fluoride was omitted; 0.5 ml of a Sephadex-treated extract was the source of enzyme (5.7 mg of protein).*

relationship between the amount of ATP added and CO₂ fixed.

Sulfite-dependent CO₂ fixation. If the system of Peck and Fisher (1962) can be coupled to the CO₂-fixing system just described, it should be possible to study the simultaneous and sequential coupling of sulfite-dependent phosphorylation with CO₂ fixation, as well as the particulars of their respective interrelationships. Simultaneous coupling will be used to describe the overall system when phosphorylation and CO₂ fixation are proceeding at the same time. Sequential coupling will refer to the system when phosphorylation and CO₂ fixation are separated in time; i.e., SO_3^- is incubated with extract, air, AMP, and P_i, and, subsequently, the system is made anaerobic to stop the phosphorylation prior to the addition of R-5-P and $C^{14}O_2$ to the system.

In the initial experiments, it was not possible to demonstrate any significant coupling between the substrate phosphorylation and the CO₂-
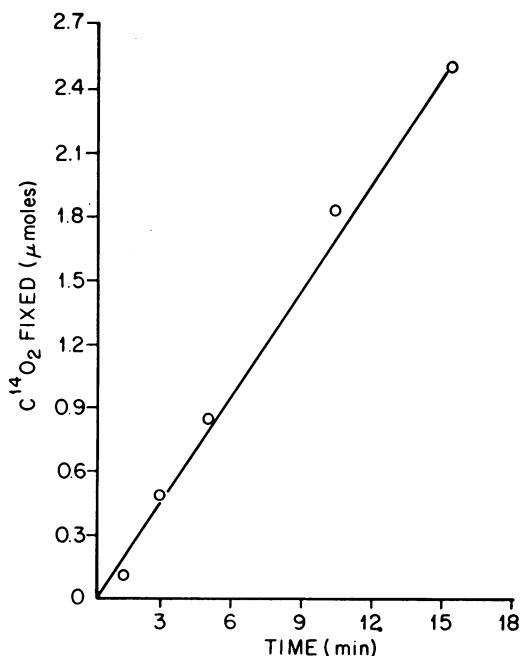
fixing enzymes, either simultaneously or sequentially. The reaction mixture for the substrate phosphorylation contained a high concentration of NaF (5×10^{-2} M) and AMP (5×10^{-3} M), both of which might interfere with the optimal operation of the CO₂-fixing system. Sulfite, the other component of the reaction mixture, was readily demonstrated to have no effect on CO₂ fixation in these extracts. As the role of AMP in the coupling of phosphorylation and CO₂ fixation should be catalytic, the AMP concentration was reduced to 5×10^{-4} M. Although NaF is required for maximal activity of the substrate phosphorylation, it was previously shown to inhibit CO₂ fixation (Table 1). Consequently, the concentration of NaF that would allow phosphorylation without radically reducing the amount of CO₂ fixed was determined. In addition, for the demonstration of significant coupling, either simultaneous or sequential, it was necessary to treat extracts with $MnCl_2$ (0.02 M, final concentration) to reduce the endogenous CO₂-fixing activity. The precipitate formed by $MnCl_2$ was removed by centrifugation, and the extract was passed over a Sephadex column, as described in Materials and Methods. This treatment

probably removed ribonucleic acids, thereby preventing the endogenous formation of high-energy phosphate by the action of polynucleotide phosphorylase. Under the conditions shown in Table 3, it was possible to partially replace added ATP by the phosphorylation accompanying the oxidation of sulfite.

For the simultaneous system, both the sulfite and CO_2-fixing components were added after the equilibration period, and the activities were allowed to proceed in air for 10 min at 30 C. For the sequential system, the oxidation of sulfite and the phosphorylation events were allowed to proceed in air at 30 C for 10 min. The air was then displaced with helium, and the components for CO_2 fixation were tipped in from the second side arm and allowed to incubate 10 min at 30 C.

The total activity under these conditions, both endogenous and exogenous, was greater when the events occurred simultaneously than when they occurred sequentially; however, the increase over the endogenous was similar in each case. The most striking result was the low activity of the coupled system which was less than 10% of the activity observed with ATP and considerably less than the activity of the substrate phosphorylation. This low activity may have been due to the hydrolysis of ATP; however, this did not

seem to be the explanation, because the amounts of CO_2 fixed in the simultaneous and sequential experiments were almost identical. Furthermore, increasing amounts of NaF did not appreciably affect the amount of CO_2 fixed in the presence of SO_3^-, although it did decrease the endogenous fixation of CO_2 (Table 4). These results suggested that some component of the reaction mixture was inhibiting either the phosphorylation or CO_2 fixation.

A critical relationship between AMP stimulation and inhibition of the sulfite-dependent $C^{14}O_2$ fixation is apparent from the evidence presented in Table 5. The small amount of endogenous activity in the absence of AMP was stimulated by the addition of 1 μmole of AMP, but severely inhibited (95%) by the presence of 5 μmoles of AMP. Likewise, the sulfite-dependent $C^{14}O_2$ fixation was stimulated approximately threefold by the presence of 1 μmole of AMP, but the presence of 5 μmoles of AMP produced almost complete inhibition of the coupling. Because of the delicate balance between the level of AMP which is required and that which inhibits, it was concluded that the probability was small of making the coupled system as active as the ATP-promoted system.

Effect of AMP on the CO_2-fixing system. The effect of increasing concentrations of AMP on the ATP-dependent CO_2 fixation, under conditions where ATP is not limiting CO_2 fixation (as evidenced by the fact that only 0.15 μmole of the available 1 μmole was used in the control), is shown in Table 6. Inhibition of CO_2 fixation was evident at an AMP-ATP ratio of 1; at a ratio of 5:1, activity was almost completely inhibited. The addition of ADP to this system did not in-

TABLE 3. *Fixation of $C^{14}O_2$ coupled to the oxidation of sulfite in a cell-free system from Thiobacillus thioparus*

System*	Oxygen uptake	$C^{14}O_2$ fixed
	μliters	μmoles
Endogenous ($-Na_2SO_3$)		
Simultaneous..........		0.08
Sequential............	7	0.04
Exogenous ($+Na_2SO_3$)		
Simultaneous..........	20	0.17
Sequential............	32	0.12

* The complete system contained in μmoles: Tris (pH 8.0), 330; AMP, 1; Na_2HPO_4, 5; EDTA, 10; NaF, 10; Na_2SO_3, 10; $MgCl_2$, 60; R-5-P, 10; $Na_2C^{14}O_3$, 5 (10^6 counts per min per μmole); 1 ml of Sephadex-treated extract (18 mg of protein). Incubation was at 30 C for 10 min in air for the simultaneous system, into which both the sulfite and CO_2-fixing components were added at the same time. In the sequential system, the oxidation of sulfite was allowed to proceed in air at 30 C for 10 min. The air was then displaced with helium, and the components for CO_2 fixation were tipped in from the second side arm and allowed to incubate for 10 min at 30 C. The reaction was stopped with 0.3 ml of 10% perchloric acid.

TABLE 4. *Effect of fluoride on the fixation of $C^{14}O_2$ coupled to the oxidation of sulfite*

System*	NaF	Oxygen uptake	$C^{14}O_2$ fixed
	μmoles	μliters	μmoles
Minus Na_2SO_3	0		0.03
	5		0.01
	10		0.01
	20		0.01
Plus Na_2SO_3	0	34	0.21
	5	26	0.15
	10	34	0.15
	20	29	0.16

* The components were the same as in Table 3, except for the variation in fluoride concentration; 1 ml of a Sephadex-treated extract (20 mg of protein) was used. Incubation time was 10 min at 30 C in air for the oxidation, and 10 min at 30 C under helium for the CO_2 fixation.

hibit CO_2 fixation, and ADP was actually capable of supporting CO_2 fixation in the absence of ATP. Since ADP increased the extent of CO_2 fixation in the presence of rate-limiting concentrations of ATP, the inhibition appeared to be specific for the mononucleotide.

Inhibition was not due to the conversion of ATP to ADP by the action of adenylic kinase, since chromatography of reaction mixtures, completely inhibited by AMP, indicated that ATP was still present and little or no ADP had been formed. In the inhibited system, essentially all of the ATP originally added could be recovered, and in the uninhibited system a little less than half of the ATP had disappeared. These results, plus the fact that ADP will support CO_2 fixation, demonstrated that the AMP inhibition of CO_2 fixation is not due to the removal of ATP by the action of adenylic kinase.

As ATP is probably present only in limiting concentrations in the coupled system, it was of interest to determine whether AMP also inhibited CO_2 fixation under conditions where ATP was limiting. The data presented in Table 7 indicate that the pattern of inhibition is the same as that obtained with nonlimiting concentrations of ATP, except that inhibition is evident at a slightly lower ratio of AMP-ATP. This result again indicates the difficulty in obtaining an active, coupled system, due to the inhibitory action of AMP.

Although a number of nucleotide triphosphates are capable of supporting CO_2 fixation (Table 8), only AMP, of the mononucleotides tested, inhibited CO_2 fixation. The specificity of the inhibition indicated that the inhibition does not result from binding of Mg^{++}, or possibly other components, by the added nucleotide. In agreement with this conclusion was the additional observation that added Mg^{++} does not relieve the

TABLE 5. *AMP inhibition of $C^{14}O_2$ fixation coupled to the oxidation of sulfite*

System*	AMP	Oxygen uptake	$C^{14}O_2$ fixed
	$\mu moles$	$\mu liters$	$\mu moles$
Minus Na_2SO_3	0.0	2	0.04
	1.0	3	0.06
	5.0	3	0.00
	10.0	3	0.00
Plus Na_2SO_3	0.0	32	0.07
	1.0	31	0.20
	5.0	32	0.03
	10.0	32	0.00

* The components were the same as Table 3, except for the variation in AMP concentration; 1 ml of a Sephadex-treated extract (15.6 mg of protein) was used. Incubation time was 10 min at 30 C in air for the oxidation, and 10 min at 30 C under helium for the fixation.

TABLE 6. *Inhibition of ATP-dependent CO_2 fixation by AMP*

ATP	AMP	$C^{14}O_2$ fixed
$\mu moles$	$\mu moles$	$\mu moles$
0.0	0.0	0.02
1.0	0.0	0.15
1.0	0.5	0.15
1.0	1.0	0.13
1.0	2.0	0.12
1.0	5.0	0.04
1.0	10.0	0.01

* The complete system was the same as in Fig. 1; 0.2 ml of a Sephadex-treated extract (1.7 mg of protein) was used.

TABLE 7. *Inhibition of ATP-dependent CO_2 fixation by AMP with limiting concentrations of ATP*

ATP	AMP	$C^{14}O_2$ fixed
$\mu moles$	$\mu moles$	$\mu moles$
0.0	0.0	0.03
0.5	0.0	0.51
10.0	0.0	2.24
0.5	0.25	0.42
0.5	0.5	0.33
0.5	1.0	0.17
0.5	2.0	0.06
0.5	5.0	0.05

* The complete system was the same as in Fig. 1; 0.5 ml of a Sephadex-treated extract (4.2 mg of protein) was used.

TABLE 8. *Mononucleotide specificity of the inhibition of CO_2 fixation*

Addition	Per cent inhibition of CO_2 fixation	
	5 μmoles	10 μmoles
AMP..................	90	95
IMP..................	13	6
GMP..................	5	20
CMP..................	9	7
UMP..................	12	7
Adenosine.............	11	15
Adenine...............	13	12

* The reaction mixture was the same as described in Fig. 1, except that 1 μmole of ATP was employed. Inhibitors were added at concentrations of 5 and 10 μmoles; 0.3 ml of a Sephadex-treated extract (2.3 mg of protein) was used.

AMP inhibition. This specific inhibition of a major energy-utilizing system by AMP may indicate that the level of AMP in these cells controls the activity of the CO_2-fixing enzymes, and, consequently, the size of the ATP pool. Since the utilization of ATP for all other syntheses must await CO_2 fixation, which provides the organic precursors for all other syntheses in an autotroph, it is possible that uncontrolled CO_2 fixation and the concomitant decrease in the ATP pool could limit the available ATP for other essential anabolic reactions. This could be especially critical in the absence of phosphorylation to restore the diminishing ATP pool. The proper balance between the various ATP-requiring activities would be expected to play a significant role in the well-being of the cell. If so, then this AMP control of CO_2 fixation could have a functional significance through its influence in maintaining the necessary energy balance required for the survival of the cell.

Sequential coupling of phosphorylation and CO_2 fixation. To measure the changes of high-energy phosphate in the absence of $C^{14}O_2$ fixation and in the presence of $C^{14}O_2$ fixation, a series of experiments with $P^{32}O_4^=$ and $C^{14}O_2$ were carried out with the coupled system. To measure the esterification of phosphate, duplicate reactions were run, and esterified phosphate was determined before and after CO_2 fixation. For the measurement of the esterification of $P^{32}O_4^=$ before CO_2 fixation, the extract was incubated for 10 min in air in the presence of $SO_3^=$, $P^{32}O_4^=$, and AMP; esterified phosphate was then determined. For the measurement of the esterification of $P^{32}O_4^=$ after CO_2 fixation, $SO_3^=$, $P^{32}O_4^=$, and AMP were incubated with the extract for 10 min in air at 30 C. After 10 min, the air was displaced with helium, R-5-P plus $Na_2C^{14}O_3$ was added, and $C^{14}O_2$ fixation was allowed to proceed for 10 min. A third set of reaction mixtures identical to those just described, but with unlabeled phosphate, was treated the same as the set with $P^{32}O_4^=$ and $Na_2C^{14}O_3$ to measure the $C^{14}O_2$ fixed. In the absence of CO_2 fixation, 172 mμmoles of phosphate were esterified, and the esterification was dependent on both sulfite and AMP as shown in column 1 of Table 9. When CO_2 was being fixed, 68 mμmoles of this labeled phosphate disappeared from the pool of high-energy phosphate (Table 9, column 2). In the absence of sulfite, there was actually a slight increase in the labile phosphate pool that may have been due to a reaction between high-molecular-weight endogenous polyphosphates or ribonucleic acids and AMP. In the absence of AMP, this could not occur, and, consequently, there was no change in

TABLE 9. *Sequential formation and utilization of high-energy phosphate for CO_2 fixation*

System*	Total labile phosphate	Δ labile phosphate	CO_2 fixation
	minus CO_2	plus CO_2	
	mμmoles	mμmoles	mμmoles
Complete...............	172	−68	84
Minus Na_2SO_3..........	63	+23	15
Minus AMP..............	57	0	20

* The complete system contained in μmoles: Tris (pH 8.0) 330; EDTA, 10; NaF, 10; $MgCl_2$, 30; $Na_2HP^{32}O_4$, 5 (4 × 10⁵ counts per min per μmole); Na_2SO_3, 10; 0.5 ml of a Sephadex-treated extract (8.7 mg of protein). For CO_2 fixation, 10 μmoles of R-5-P and 5 μmoles of Na_2CO_3 (10⁶ counts per min per μmole) were added. The flasks were incubated in air for 10 min, flushed with He for 5 min, and, for CO_2 fixation, incubated under He for 10 min with R-5-P, 10 μmoles, and $Na_2C^{14}O_3$, 5 μmoles (10⁶ counts per min per μmole). Labile phosphate was measured as $P^{32}O_4$, which was adsorbable on acid-washed charcoal and hydrolyzable by treatment with 1 N HCl at 100 C for 15 min.

the pool under these conditions. The data in column 3 (Table 9) show the amount of $C^{14}O_2$ fixed during the utilization of high-energy phosphate indicated in column 2. If one applies a correction for the largest amount of $C^{14}O_2$ fixed in the absence of AMP, the amount of $C^{14}O_2$ fixed is 64 mμmoles when 68 mμmoles of high-energy phosphate were utilized. Or, if one makes a correction for the total high-energy phosphate involved and total $C^{14}O_2$ fixed, the values are 91 mμmoles of high-energy phosphate and 84 mμmoles of $C^{14}O_2$. This stoichiometry is practically identical with the theoretical requirement beginning with R-5-P.

The $P^{32}O_4^=$ esterified during the oxidation was found—by paper electrophoresis and chromatography, followed by radioautography—to be mainly present in both ADP and ATP. In agreement with the mechanism of Peck and Fisher (1962), the larger amount of label was found in the ADP.

DISCUSSION

Whether substrate phosphorylation (Peck and Fisher, 1962) is the only mechanism for production of high-energy phosphate in this organism is as yet an unanswered question. That it might satisfy the energetic requirements of the cell has been suggested by Peck (1962); however, on the

basis of theoretical ATP requirement and observed cell yield, it has been indicated that this substrate phosphorylation cannot supply all the energy for growth (Vishniac and Trudinger, 1962). This fact—plus the known existence of cytochromes (Cook and Umbreit, 1963; Trudinger, 1961; Aubert et al., 1958), coenzyme Q (Cook and Umbreit, 1963), and the inhibition of CO₂ fixation and ATP formation in T. thioparus by 2,4-dinitrophenol (Kelley and Syrett, 1963; Kelley and Syrett, 1964)—suggests the existence of oxidative phosphorylation in these organisms, but, as yet, no direct evidence has been presented. In any case, the phosphorylating system of Peck and Fisher (1962) and the CO₂-fixing system described here provide the minimal requirements for studies on the relationship between phosphorylation and CO₂ fixation, particularly the sequential coupling of phosphorylation and CO₂ fixation.

Although considerable discussion has persisted over the significance of the data presented by Umbreit and co-workers concerning the separation in time of phosphorylation and CO₂ fixation in T. thiooxidans, the generalizations made from these data have been substantiated in other organisms. It is not intended to discuss the controversy here, as this aspect has recently been ably reviewed by Larsen (1960). The demonstration that, in extracts, a phosphorylation involved in the oxidation of reduced sulfur compounds to $SO_4^=$ can be stoichiometrically coupled with CO₂ fixation supports the applicability of the generalization concerning separation in time of phosphorylation and CO₂ fixation to the thiobacilli, albeit the present work was done with T. thioparus and a different substrate.

Ru-1,5-diP carboxydismutase is the critical enzyme involved in autotrophic CO₂ fixation. Analysis of metabolites after short exposure of cells of T. denitrificans to $C^{14}O_2$ indicates that the Calvin cycle functions in these organisms (Milhaud, Aubert, and Millet, 1956). Furthermore, data indicating the presence of carboxydismutase in extracts of T. thioparus, T. denitrificans, and T. thiooxidans have been published; however, specific activities were either low or could not be calculated. The specific activity of the CO₂-fixing system reported here, approaching that of green plants, is further evidence for the importance of the role of carboxydismutase in thiobacilli.

It is not known whether the utilization of all the nucleoside triphosphates tested for CO₂ fixation is a reflection of nucleotide nonspecificity, or an indication of kinase activity allowing for the transfer of phosphate from all of the nucleoside triphosphates tested to AMP or ADP. A distinction between these two possibilities will not be possible until the phosphoribulokinase is sufficiently purified to test directly the nucleotide specificity. Purification of this enzyme and the carboxydismutase is underway.

Autotrophic organisms, by definition, are capable of synthesizing their total cellular material from CO₂ and are, therefore, considered to be the most biosynthetically complete of known organisms. Imposed on this biosynthetic metabolism is an energy-producing system, chemosynthetic or photosynthetic, and a very potent CO₂-fixing system. The CO₂-fixing system must utilize a comparatively large fraction of the ATP produced by the cell and might be thought of as an "ATP-sink." During periods of energy deprivation, this system, uncontrolled, could rapidly deplete accumulated ATP and endogenous energy reserves, and possibly result in the premature death of the cells. Thus, it might be postulated that there exists a mechanism for controlling CO₂ fixation, and thereby the level of intracellular ATP during periods of energy shortage or deprivation.

The inhibition of the CO₂-fixing system by AMP may represent such a control mechanism for maintaining a critical level of ATP. If the in vitro results are applicable to the intact cells, when 65 to 70% of the adenosine nucleotides are in the form of AMP, the CO₂-fixing system is completely inhibited, thus permitting the cells to maintain the level of ATP necessary for other essential reactions in the cell. This inhibition does not represent a Mg^{++}-AMP effect (Lardy and Parks, 1956), and it is highly specific for AMP—other nucleotides and ADP being ineffective. Further studies will be required to determine the locus of the inhibition, the type of inhibition, and the composition of the adenosine nucleotide pool in whole cells under conditions of energy deprivation.

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