JOURNAL OF BACTERIOLOGY
Vol. 88, No. 4, p. 850-857 October, 1964


# EFFECT OF PHOSPHATE ION AND 2,4-DINITROPHENOL ON THE ACTIVITY OF INTACT CELLS OF *THIOBACILLUS FERROOXIDANS*

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Received for publication 6 April 1964

## ABSTRACT

BECK, JAY V. (Brigham Young University, Provo, Utah), AND FRED M. SHAFIA. Effect of phosphate ion and 2,4-dinitrophenol on the activity of cell suspensions of *Thiobacillus ferrooxidans*. J. Bacteriol. **88**:850-857. 1964.—The rate of oxidation of ferrous salts or elemental sulfur by aged cell suspensions, phosphate-depleted cells, or 2,4-dinitrophenol (DNP)-treated cells of *Thiobacillus ferrooxidans* was increased by addition of orthophosphate salts. The effect was found to be transitory, with the rate gradually approaching that observed prior to phosphate ion addition. The total increased oxygen uptake was observed to be roughly proportional to the amount of phosphate salt added. The efficiency of $CO_2$ fixation accompanying oxidation of ferrous salts was found to be about 1.7 $\mu$moles of $CO_2$ fixed per 100 $\mu$moles of $O_2$ absorbed, in contrast to a value of about 8.0 $\mu$moles of $CO_2$ fixed per 100 $\mu$moles of $O_2$ uptake during sulfur oxidation. The rate of oxidation did not affect the $CO_2$ fixation efficency. Whereas addition of phosphate salts to aged or phosphate-depleted cells increased slightly the already high efficiency of $CO_2$ fixation, it did not affect the complete inhibition of $CO_2$ fixation observed in the presence of $10^{-5}$ M DNP. The results indicate that the phosphate ion is essential for oxidation of the ferrous ion, and that dinitrophenol and other so-called uncoupling agents interfere with phosphate metabolism. The latter may be a result of action at the site of assimilation of the ferrous ion or it may be an effect on the electron-transport system. In any event, it seems obvious that the phosphate ion is converted into a nonactive form in the presence of dinitrophenol-treated cells, because additional quantities of orthophosphate salts cause an immediate, marked restoration of oxidative activity.

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The ability of intact cells of an acidophilic iron-oxidizing bacterium to oxidize either ferrous ion or elemental sulfur in manometric experiments has been reported (Silverman and Lundgren, 1959; Beck, 1960). Carbon dioxide fixation was demonstrated to accompany the oxidation of each substrate. The effects of orthophosphate and 2,4-dinitrophenol (DNP) on oxygen uptake and carbon dioxide fixation during ferrous iron oxidation also were reported previously (Beck and Shafia, 1960, 1961). Further studies on the interesting effects of orthophosphate and DNP on the activity of intact cells of *Thiobacillus ferrooxidans* are presented in this paper.

The iron-oxidizing bacterium used in this work and previously described (Beck, 1960) is similar to, and for the present will be identified as, *T. ferrooxidans*, which is probably the most appropriate name for all iron-oxidizing eubacteria which also oxidize some form of sulfur (Ivanov and Lyalikova, 1962).

## MATERIALS AND METHODS

*Cell growth.* Intact cell suspensions for the earlier work were obtained as previously reported (Beck, 1960). More recently, a method using continuous growth has been adopted. The growth vessel consisted of a 12-liter Pyrex bottle fitted with a rubber stopper to admit the culture medium input tube, an overflow tube, and a sintered-glass sparger. The volume of culture in the growth vessel was maintained at 10 liters by placing the end of the overflow tube at the 10-liter mark. The aeration gas, compressed air with or without added carbon dioxide, forced the spent culture through the overflow tube and into a 20-liter collection bottle, which, upon being filled, was stored at 4 C. Cells were harvested with a Sharples centrifuge. The cell suspensions were cleared of insoluble ferric compounds by

differential centrifugation. The cells were washed at least twice with, and then suspended and stored in, distilled water at 2 to 4 C.

The culture medium consisted of $FeSO_4 \cdot 7H_2O$, 4%; $(NH_4)_2SO_4$, 0.2%; $KH_2PO_4$, 0.01%; $MgSO_4 \cdot 7H_2O$, 0.04%; pH 2.9 (with 10 N $H_2SO_4$). The redox potential of the culture medium was initially 536 mv and after growth, 775 mv. The pH of the medium was unchanged by growth of the bacterium. Ferrous sulfate concentrations as high as 8% and pH values as low as 1.8 are tolerated by the bacterium, but with a decreased rate of growth. The growth medium was prepared with tap water and was not sterilized. Frequent observations by direct microscopic examination and by plating on neutral or acidic nutrient agar indicated no contamination of the culture. Incubation was at room temperature, which varied from 23 to 26 C.

The culture medium was fed by gravity or by pump into the growth vessel at the rate of about 500 ml/hr. Since aeration of the growth vessel contents caused rapid mixing as the culture medium was introduced, the ferrous ion was rapidly oxidized by the culture and the redox potential remained constant at 775 mv. Cell production, therefore, occurred under conditions of constant pH and constant redox potential. Cell yield was noted to be about $2 \times 10^8$ cells per ml or $3 \times 10^8$ cells per ml if $CO_2$ was fed into the growth vessel with the aeration gas. Cellular nitrogen values varied from 0.75 to 1.0 mg per liter in the presence of added carbon dioxide.

*Carbon dioxide fixation.* Manometric experiments were performed by use of a conventional Warburg apparatus. Fixation of $CO_2$ into intact cells was determined after addition of $Na_2C^{14}O_3$ from a side arm of a Warburg vessel. At the conclusion of the Warburg run, 0.5 ml of 6 N HCl was added to the flask to stop the reaction and to dissolve all the precipitated iron compounds. The acidified flask contents and 2.5-ml flask washings were combined to make a total volume of 5 ml. After flushing the solution for 10 min with $C^{12}O_2$, portions of solution were placed in stainless-steel planchets for $C^{14}$ determination with the use of a thin-window flow counter. Some loss of $C^{14}$ activity was noted as a result of repeated treatment of the planchet contents with 6 N HCl. This loss was considered to be caused by decomposition or volatilization of organic compounds, or both, and not due to initial retention of $C^{14}O_2$ in the planchet residue. In sulfur experiments, samples were taken directly from the Warburg flasks, placed in planchets, acidified, dried, and counted.

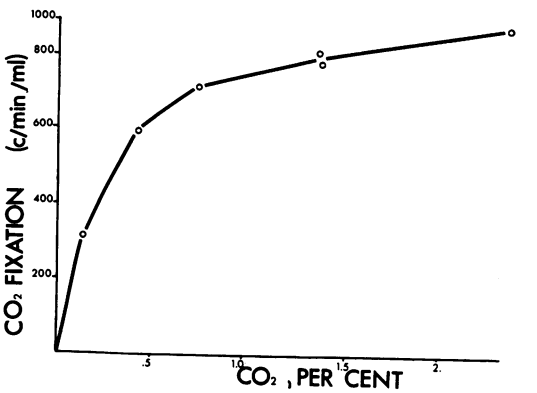


FIG. 1. *Carbon dioxide fixation by Thiobacillus ferrooxidans as affected by carbon dioxide concentration. Reaction vessels contained 100 μmoles of $FeSO_4$ (1 ml of 0.1 M $FeSO_4$ in 0.01 N $H_2SO_4$), 0.04 mg of cellular nitrogen, and 10 μmoles of $KH_2PO_4$. Total volume, 2.0 ml. Temperature, 32 C; initial pH, 2.7. $Na_2C^{14}O_3$ (13,400 counts per min per μmole), 0.05 M, was added from side arm in amounts of 1.0, 2.5, 5.0, 10.0, and 15.0 μmoles to give the approximate $CO_2$ percentages in the gaseous phase, as indicated. The $CO_2$ fixation values shown are the actual counts on an infinitely thin layer with one-fifth of the reaction vessel contents. The oxygen uptake in this experiment was 17.5 μmoles.*

The partial pressure of $CO_2$ in the reaction flask has a marked effect on $CO_2$ fixation. This is shown in Fig. 1, in which the $CO_2$ concentration, expressed as volume per cent of the gaseous phase in the reaction vessel, is plotted against the $CO_2$ fixed. The data are for $CO_2$ fixation accompanying oxidation of 100 μmoles of ferrous sulfate. The efficiency of $CO_2$ fixation may be expressed as the ratio of μmoles of $CO_2$ fixed per 100 μmoles of oxygen absorbed. Efficiency values for the data of Fig. 1 are 0.7, 1.3, 1.7, 1.9, and 2.0. Variation of the rate of oxidation or of the total oxidation, caused by changing the cell concentration or the ferrous sulfate concentration, did not affect the $CO_2$ fixation efficiency values.

## RESULTS

*Phosphate effect.* Previously reported manometric experiments showed the rate of ferrous ion oxidation by intact cells of *T. ferrooxidans*

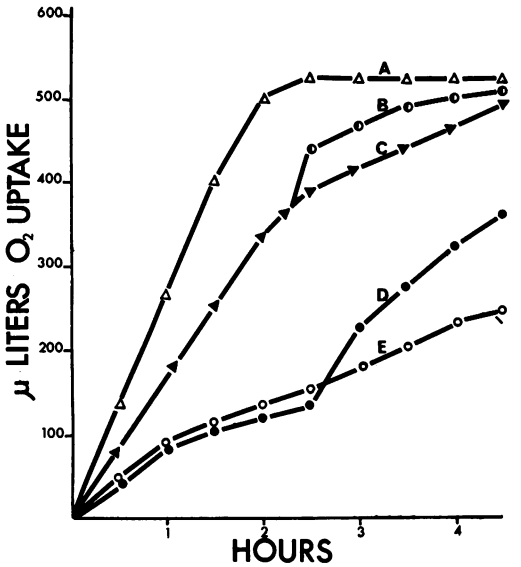


FIG. 2. *Effect of phosphate ion on the activity of cell suspensions of Thiobacillus ferrooxidans. Reaction vessels contained 0.07 mg of cellular nitrogen, 200 μmoles of $FeSO_4$, 10 μmoles of $MgSO_4$, 10 μmoles of $(NH_4)_2SO_4$, and $KH_2PO_4$ as indicated in a total volume of 2.0 ml. Temperature, 32 C; pH, 2.8. Symbols:* ○, *phosphate-depleted cells (see text);* ●, *phosphate-depleted cells + 10 μmoles of $KH_2PO_4$ added from side arm at 140 min;* ▲, *aged cells;* ◐, *aged cells + 10 μmoles of $KH_2PO_4$ added from side arm at 130 min;* △, *fresh cells.*

to be constant and independent of the substrate concentration until near exhaustion of the substrate. Subsequent experiments with cell suspensions stored for extended periods at 4 to 6 C showed decreasing rates of oxidation considerably before the substrate was completely utilized. In attempts to restore such cells to the behavior of fresh suspensions, the components of the growth medium were added to aged suspensions in manometric experiments. Interestingly, marked stimulation of activity was observed only upon addition of $KH_2PO_4$. The stimulating effect of phosphate is shown in Fig. 2. Freshly harvested cells were used to obtain data shown as curve A; cells stored in distilled water at 4 C for 25 days were used for curves B and C; and phosphate-depleted cells, obtained by incubation for 8 hr in presence of ferrous sulfate and absence of phosphate, were used for curves D and E. Similar results were consistently noted in many experiments. Magnesium sulfate or ammonium sulfate, or both, had no effect on the rate of activity nor on the phosphate effect. Freezing of cell suspensions greatly reduced their rate of iron oxidation, and the addition of orthophosphate caused a partial restoration of activity.

The relationship between the quantity of added phosphate and the resulting increased oxygen absorption is shown in Fig. 3 and 4. The cells used in these experiments were preincubated in a phosphate-free, ferrous sulfate solution at 30 C for 30 min. Upon addition of phosphate, there was a marked increase in the rate of oxygen uptake, which thereafter slowly decreased and approached the rate existing prior to phosphate addition (Fig. 3). In this experiment, the ferrous salt was completely oxidized before the phosphate became limiting at the two higher phosphate levels. The differences of oxygen absorption after phosphate addition are shown in Fig. 4. At phosphate levels of 6, 9, and 20 μmoles, there was observed a return to the prephosphate rate of oxidation and, at higher phosphate levels, the

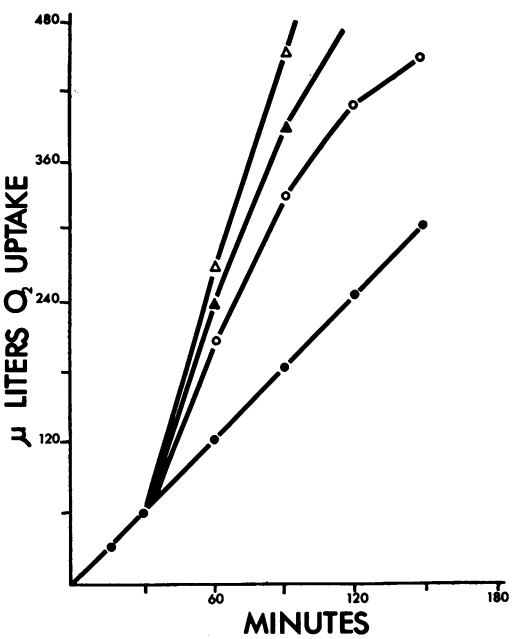


FIG. 3. *Effect of phosphate ion concentration on the rate of ferrous ion oxidation by cell suspensions of Thiobacillus ferrooxidans. Reaction vessels contained 200 μmoles of $FeSO_4$, 10 μmoles of $MgSO_4$, 10 μmoles of $(NH_4)_2SO_4$, 0.135 mg of cellular nitrogen (phosphate-depleted cells), and $K_2HPO_4$ as indicated in a total volume of 2.0 ml. Temperature, 32 C; pH 2.8. Symbols:* ●, *no phosphate;* ○, *20 μmoles of $KH_2PO_4$;* ▲, *40 μmoles of $KH_2PO_4$;* △, *80 μmoles of $KH_2PO_4$.*

prephosphate rate was approached. The relationship between added phosphate and increased oxygen absorption may be expressed as a P/O ratio. This value is shown in Table 1 for data obtained in three experiments. Although the observed ratios are not constant, they do tend to be about 1.

These results show the dependency of the rate of iron oxidation on the concentration of orthophosphate. They suggest that the phosphate ion is essential for iron oxidation in aged or frozen cells of *T. ferrooxidans*, and that it is converted into some form which is not readily available to the oxidizing system. The latter seems a likely explanation of the observation that a second addition of phosphate causes a second rate increase in oxygen absorption.

*Effect of DNP.* The dependence of the rate of ferrous ion oxidation on phosphate suggested an obligatory phosphorylation reaction accompanying electron transport. It was expected, therefore, that so-called uncoupling agents would stimulate the oxidation reaction. Instead, DNP concentrations normally stimulatory to mitochondrial preparations ($10^{-4}$ M) inhibited the rate of iron oxidation by fresh cell suspensions of *T. ferrooxidans* (Fig. 5). Addition of inorganic orthophosphate stimulated the rate of oxygen absorption by cell suspensions whose oxidative activity had almost ceased as a result of DNP inhibition. As was noted with aged or frozen cells, the amount of increased oxygen uptake was found to be dependent on the amount of phosphate ion added, being about 1 mole of oxygen for each 2 moles of orthophosphate added, or a P/O ratio of about 1. The data show that a second addition also stimulated the rate of oxygen absorption, and further support the idea that free orthophosphate, essential for iron oxidation by intact cells, in the course of iron oxidation in the presence of DNP is converted into some form not readily available to the cells. Preincubation of cells for 30 min to 1 hr at 30 C with or without orthophosphate or DNP, or both, did not affect the rate at which they were inactivated by DNP in phosphate-free media containing ferrous sulfate.

The stimulatory phosphate effect was observed after addition of $KH_2PO_4$, $NaH_2PO_4$, and $NH_4H_2PO_4$, although in somewhat smaller degree with the sodium salt. No stimulation was observed after addition of inorganic pyrophosphate. In the presence of high orthophosphate concen-

TABLE 1. *Relation between increased oxygen absorption and added phosphate**

| Orthophosphate added | Expt 1 | | Expt 2 | | Expt 3 | |
|---|---|---|---|---|---|---|
| | Increased oxygen absorbed | P/O | Increased oxygen absorbed | P/O | Increased oxygen absorbed | P/O |
| *μmoles* | *μmoles* | | *μmoles* | | *μmoles* | |
| 6 | 1.6 | 1.9 | | | | |
| 9 | 3.4 | 1.3 | | | | |
| 20 | | | 8.0 | 1.3 | 20.1 | 0.5 |
| 40 | | | 14.3 | 1.4 | 29.0 | 0.69 |
| 80 | | | 17.0† | 2.3† | 42.5 | 0.94 |

* Conditions same as for Fig. 2, except for experiment 3, which had 400 μmoles of $FeSO_4$ instead of 200 μmoles used in experiments 1 and 2.

† Ferrous ion was exhausted before decreased rate because phosphate deficiency occurred.

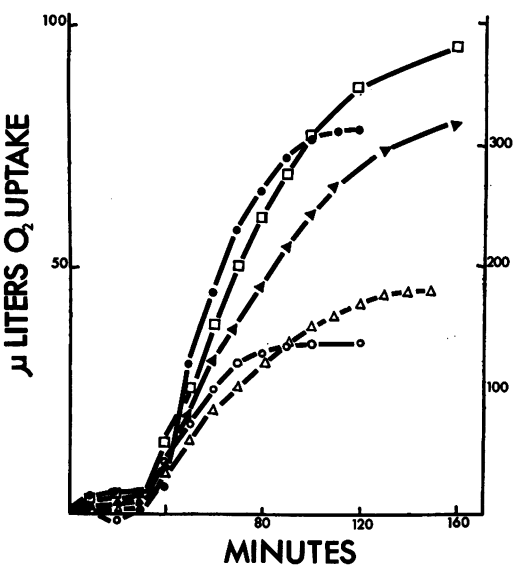


FIG. 4. *Relationship between increased oxygen uptake and phosphate ion concentration. The oxygen uptake shown is the difference between values with added $KH_2PO_4$ and the control with no added phosphate. Reaction vessels contained 200 μmoles of $FeSO_4$, 10 μmoles of $MgSO_4$, 10 μmoles of $(NH_4)_2SO_4$, and 0.135 mg of cellular nitrogen in a total volume of 2.0 ml. Temperature, 32 C; pH, 2.8. Phosphate was added at 30 min. Symbols: ○, 6 μmoles of $KH_2PO_4$, left ordinate; ●, 9 μmoles of $KH_2PO_4$, left ordinate; △, 20 μmoles of $KH_2PO_4$, right ordinate; ▲, 40 μmoles of $KH_2PO_4$, right ordinate; □, 80 μmoles of $KH_2PO_4$, right ordinate.*

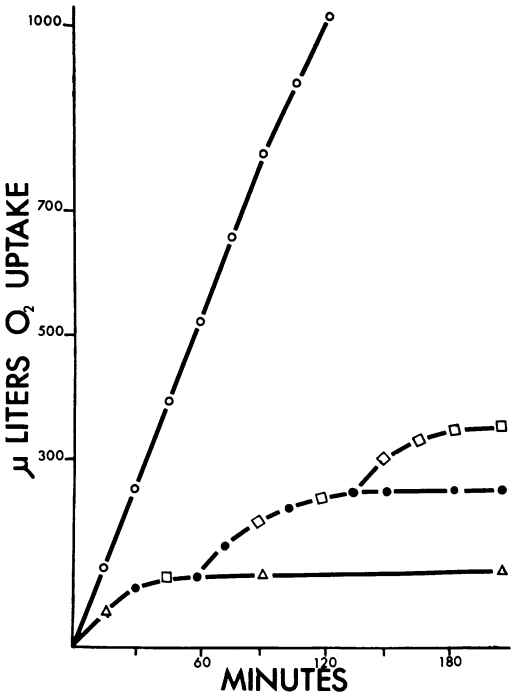


FIG. 5. *Effect of phosphate ion on the rate of oxygen absorption by 2,4-dinitrophenol-inhibited cell suspensions of Thiobacillus ferrooxidans. Reaction vessels contained 200 μmoles of $FeSO_4$ and 0.09 mg of cellular nitrogen in a total volume of 3.0 ml. Temperature, 32 C; pH, 2.8. Symbols: ○, nothing added; ●, △, □, 0.3 ml of $10^{-3}$ M DNP added; 10 μmoles of orthophosphate added to ● and □ at 60 min, and an additional 10 μmoles to □ at 135 min. Although not shown, the reaction ○ went to completion with total $O_2$ uptake of 1,220 μliters at 200 min.*

trations, the nucleotides adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate (ATP) were slightly inhibitory, whereas in the absence of added orthophosphate each nucleotide was slightly stimulatory. The latter effect may have been due to slow degradation of the nucleotides in the highly acidic environment, thus yielding inorganic phosphate.

Some other so-called uncoupling agents also inhibited the rate of iron oxidation by intact cells (Fig. 6), and their effect was reversed upon addition of phosphate ion. On the other hand, the strongly inhibitory action of $2 \times 10^{-5}$ M *m*-chlorocarboxylcyanide phenylhydrazone, reported to be a vigorous uncoupling agent (Heytler et al., 1962), could not be reversed in the presence of inorganic orthophosphate.

The oxidation of sulfur by cell suspensions was inhibited in the presence of $10^{-4}$ M DNP. Phosphate addition in the presence of DNP caused an increased rate of oxidation, but the relationship between increased oxygen absorption and the phosphate added has not been established in the case of sulfur oxidation.

Several common inhibitors of oxidative processes are effective against intact cells of *T. ferrooxidans*. The following were found to completely inhibit ferrous ion oxidation at the indicated concentrations: sodium azide, $10^{-3}$ M; potassium cyanide, $10^{-3}$ M; sodium sulfide, $10^{-3}$ M. Antimycin A ($10^{-4}$ M) and chloramphenicol ($10^{-3}$ M) showed no effect on either iron or sulfur oxidation.

Attempts to identify the unavailable form of phosphate have been disappointing. Considerable difficulty arises because of the insolubility of ferric phosphate at the pH values used in these experiments. Any organic or polymerized phosphate formed may also be quite insoluble. Thus, one

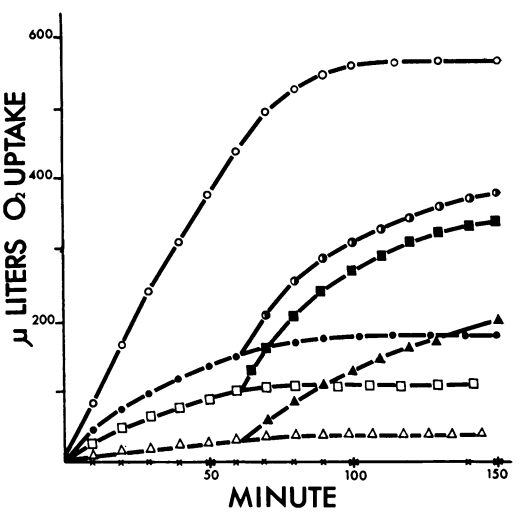


FIG. 6. *Effect of several inhibitors and phosphate ion on the activity of intact cell suspensions of Thiobacillus ferrooxidans. Reaction vessels contained 100 μmoles of $FeSO_4$, 0.09 mg of cellular nitrogen, and inhibitor and phosphate as indicated in 2.7 ml total volume. Temperature, 32 C; pH, 2.8. Cells (0.3 ml) and inhibitor (0.4 ml) were incubated in side arm for 25 min, and were then added to the substrate at 0 time; 20 μmoles of $KH_2PO_4$ (pH 2.0) were added at 60 min. Symbols: ○, no inhibitor, no $KH_2PO_4$; ×, 0.02 M sodium azide; △, 0.005 M dichlorphenoxy acetic acid (DPAA); ▲, DPAA + $KH_2PO_4$; □, 0.0001 M dinitrocresol (DNC); ■, DNC + $KH_2PO_4$; ●, 0.001 M 2,4-dinitrophenol (DNP); ◐, DNP + $KH_2PO_4$.*

cannot simply centrifuge the Warburg vessel contents to determine uptake of phosphate by the cells. Although detailed experimental procedures and results will be presented in another report, some general observations may be pertinent here. Distribution of added orthophosphate-$P^{32}$ in the supernatant fluid, residue, or residue extracts from reaction solutions was similar whether or not DNP was present during the reaction. Citric acid (0.01 M, pH 4.2) extracts of the cell residue from reaction vessels containing DNP and added phosphate showed absorption maxima at 250 to 260 mμ. This was not observed in the absence of added phosphate. Also, the development of color in the Fiske-SubbaRow method for inorganic phosphate was slightly delayed in solutions from reaction vessels in which phosphate had been shown to stimulate the rate of oxidation in the presence of DNP.

Sodium arsenate ($10^{-3}$ M) in the absence of added phosphate restores activity to both aged cell suspensions and DNP-inhibited suspensions, and slightly enhances the above-noted effects of phosphate additions.

*Carbon-dioxide fixation.* Recent experiments confirmed earlier observations that intact cells of *T. ferrooxidans* are capable of fixing about 2.0 μmoles of $CO_2$ per 100 μmoles of oxygen used in oxidizing ferrous iron. The corresponding value accompanying sulfur oxidation has been found to be about 8.0 μmoles of carbon dioxide per 100 μmoles of oxygen. The latter value is much higher than that previously reported for sulfur oxidation alone, but agrees well with the value attributed to sulfur oxidation on a mixed sulfur-ferrous iron substrate (Beck, 1960).

The effect of DNP on the rate of oxidation and the efficiency of carbon dioxide fixation, expressed as μmoles of $CO_2$ absorbed per 100 μmoles of $O_2$ uptake, accompanying either ferrous ion or sulfur oxidation by intact cells of *T. ferrooxidans* is shown in Table 2. A marked similarity is noted in effect of DNP on both oxygen and $CO_2$ absorption with either iron or sulfur as the oxidizable substrate. At a concentration of $10^{-6}$ M DNP, there was observed about 50% inhibition of the oxidation rate, but no effect on $CO_2$ fixation. As the DNP concentration increased, there occurred a gradually increasing inhibitory effect on oxidation and a more rapidly increasing inhibition of $CO_2$ fixation efficiency until, at a DNP concentration of $10^{-5}$ M, the $CO_2$ fixation was completely inhibited and the inhibition of oxidation reached 80 to 90%. Over 99% inhibition of oxidation was observed at $10^{-4}$ M DNP.

TABLE 2. *Effect of 2,4-dinitrophenol on the activity of intact cells of Thiobacillus ferrooxidans**

| DNP | Sulfur oxidation | | Ferrous ion oxidation | |
|---|---|---|---|---|
| | $Q_{O_2}$ (nitrogen) | $CO_2$/100 μmoles of $O_2$ | $Q_{O_2}$ (nitrogen) | $CO_2$/100 μmoles of $O_2$ |
| *M* | | *μmoles* | | *μmoles* |
| 0 | 500 | 8.2 | 10,000 | 2.1 |
| $10^{-6}$ | 240 | 8.5 | 5,000 | 2.8 |
| $2 \times 10^{-6}$ | 210 | 7.3 | | |
| $4 \times 10^{-6}$ | 180 | 3.0 | | |
| $6 \times 10^{-6}$ | 100 | 0 | 4,000 | 0.20 |
| $10^{-5}$ | 40 | 0 | 2,000 | 0 |
| $10^{-4}$ | 1–2 | 0 | 80 | 0 |

* Reaction vessels contained 0.06 mg of cellular nitrogen, 100 μmoles of $FeSO_4$ or 100 mg of sulfur, and 10 μmoles of $KH_2PO_4$ in a total volume of 2.0 ml. Temperature, 32 C; pH, 2.8.

As discussed above, addition of small amounts of phosphate caused a marked, but transitory, increase in the rate of oxidation of ferrous iron or sulfur by intact cells in the presence of $10^{-5}$ M DNP, but such additions had no effect on efficiency of $CO_2$ fixation. Detectable $CO_2$ fixation has never been observed in reaction vessels containing $10^{-5}$ M DNP whether or not orthophosphate was present. Preincubation in the presence of phosphate had no effect on the ability of intact cells to fix carbon dioxide in $10^{-5}$ M DNP solutions.

The efficiency of $CO_2$ fixation during ferrous iron oxidation by aged cells is diminished along with the rate of oxidation, and may be restored to normal efficiency in the presence of orthophosphate. In one experiment, the number of μmoles of $CO_2$ fixed per 100 μmoles of $O_2$ uptake was increased from 1.2 to 1.7 by the addition of orthophosphate.

In contrast to the effect of DNP, the inhibitory action of *m*-chlorocarboxylcyanide phenylhydrazone on the oxidation of ferrous ion by intact cells of *T. ferrooxidans* was not accompanied by reduction in the efficiency of $CO_2$ fixation. Thus, at a concentration of $2 \times 10^{-5}$ M *m*-chlorocarboxylcyanide phenylhydrazone, the rate of ferrous ion oxidation was reduced 85%, whereas the efficiency of $CO_2$ fixation was normal, being 1.9

$\mu$moles of $CO_2$ fixed per 100 $\mu$moles of $O_2$ absorbed.

## DISCUSSION

The increased rate of iron oxidation by aged cells after treatment with orthophosphate was first considered to be due to removal of ferric ion as a result of chelation by the added phosphate. Although it was noted that increased ferric ion concentration inhibits the rate of growth and extends the lag period of *T. ferrooxidans* (Shafia, 1960), it is recognized that the ferric ion concentration has little effect on activity of intact cells. This is indicated by the almost linear relationship between time and $O_2$ absorption as the ratio of ferrous ion to ferric ion changes from a very high to a very low value in manometer reaction vessels. Additions of ferric sulfate initially or at a later time did not affect the rate of ferrous iron oxidation. One must conclude that the phosphate ion is directly involved in ferrous ion oxidation by intact cells either in substrate assimilation or in the actual electron-transport reactions. The remarkable effect of phosphate in the DNP inhibition experiments further suggests its direct role in the oxidative mechanisms.

A possible explanation of the phosphate effect is that orthophosphate is converted to a highly labile, 250- to 260-m$\mu$ absorbing compound, from which in normal cells it is readily released for further use. Aging or, to a greater extent, the presence of DNP prevents the release of orthophosphate and thus reduces the dependent rate of ferrous ion oxidation.

The complete repression of carbon dioxide fixation by DNP even at relatively high respiratory rates further suggests that phosphate metabolism is affected. Kelly and Syrett (1963) showed that DNP does not completely inhibit $CO_2$ fixation by *T. thioparus* at $10^{-4}$ M concentration. These observations indicate that phosphate in the presence of DNP-treated cells is no longer converted into a utilizable high-energy form, such as ATP, essential in the $CO_2$ fixation reaction. The coupling of electron transport to $CO_2$ fixation is not obligatory, as was shown earlier by using negligible $CO_2$ concentrations (Beck and Elsden, 1958) and, presently, by using DNP which interferes with phosphate metabolism.

The values for $CO_2$ fixation in sulfur oxidation presently reported are much higher than those found earlier (Beck, 1960). The reasons for this are not clear, but may be due to more complete understanding of the factors, such as $CO_2$ concentration and phosphate concentration, which affect the efficiency of $CO_2$ fixation.

The ratio of the number of $\mu$moles of $CO_2$ fixed to 100 $\mu$moles of oxygen absorbed has been shown to be about $1.8 \pm 0.2$ for oxidation of ferrous ion, and about $8 \pm 0.5$ for sulfur oxidation. Now, if carbon dioxide fixation may be used as an index of high-energy phosphate (ATP) production, then sulfur oxidation yields approximately four times as much ATP as does iron oxidation. Further, if one assumes that oxidative phosphorylation accounts for all ATP formation, as seems likely from the inhibitory action of DNP on $CO_2$ fixation and other observations (Vernon et al., 1960), and that at least 1 ATP is formed per electron pair transferred during iron oxidation, then one comes to the unlikely conclusion that 4 ATP are formed per electron pair in sulfur oxidation. A plausible explanation is that some ATP may be required in a reversed oxidative phosphorylation reaction, as discussed by Elsden (1962), which is necessary to form reduced nicotinamide adenine dinucleotide (NADH) during iron oxidation. This may require 2 ATP per mole of NADH formed (Chance and Hagihara, 1961), and would greatly decrease the apparent efficiency of $CO_2$ fixation during iron oxidation, thus causing the ratio of ATP from sulfur oxidation to that from iron oxidation to be apparently increased. Crude extracts of *T. ferrooxidans* grown on ferrous sulfate were shown in our laboratory to have NADH-mammalian cytochrome *c* reductase activity. Attempts to demonstrate an accompanying phosphorylation were inconclusive.

A substrate phosphorylation not associated with oxidation also may be a possible explanation of the above noted 4 ATP per electron pair. Although unpublished results from our laboratory and the report of Lazaroff (1963) demonstrated a dependence of iron oxidation on the presence of sulfate ions, thus suggesting an adenosine-5′-phosphosulfate-type substrate phosphorylation (Peck and Fisher, 1961), preliminary attempts to demonstrate ATP sulfurylase in crude extracts of the iron bacteria were unsuccessful.

Again, if one assumes the carbon content of cells to be 50% one may calculate, using the above $CO_2$ fixation efficiency data, that 0.11 g (dry weight) of cells is formed per mole of $FeSO_4$ oxidized. This value agrees very well with the observed 0.1 g of cells per mole of $FeSO_4$ in

growth experiments. Similarly, about 0.8 g of cells is formed per electron pair in sulfur oxidation. These values may be compared with the value of 10 g of cells per mole of ATP observed by several investigators for several bacterial types (Elsden, 1962).

ACKNOWLEDGMENTS

The excellent technical assistance of Heinz L. Hosch and Carol E. Hodgson is gratefully acknowledged. This study was aided by Public Health Service grant C1475-C8 from the National Institutes of Health and grants G11352 and G23322 from the National Science Foundation.

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