STUDIES ON THE METABOLISM OF AUTOTROPHIC BACTERIA*

II. THE NATURE OF THE CHEMOSYNTHETIC REACTION

BY K. G. VOGLER

(From the Department of Agricultural Bacteriology, University of Wisconsin, Madison)

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Chemosynthesis is defined as the synthesis of cell material from CO$_2$ by the use of chemical energy obtained in oxidation. This synthesis occurs in the dark. In the strictly autotrophic bacteria, *Thiobacillus thiooxidans*, the CO$_2$ furnishes not only the entire cell substance but is the only carbon source available for growth. The energy is provided by the oxidation of sulfur. The concept of an autotrophic bacterium which emerges from the studies described in the previous papers (Vogler *et al,* 1942; Vogler, 1942) is one in which the autotrophic cell possesses an internal organic metabolism. This is similar in some respects to that of heterotrophic organisms but differs in the inability of the cell to utilize the common substrates of heterotrophic growth. The cell possesses a mechanism of energy supply from the oxidation of sulfur and with this energy supply it can synthesize its cell materials from CO$_2$. This paper is concerned with how the cell is able to utilize CO$_2$.

Methods

The CO$_2$ fixation by *Thiobacillus thiooxidans* has been studied by a method in which very small amounts of CO$_2$ were added to a CO$_2$-free atmosphere (or air) over suspensions of the organism in Dixon-Keilin flasks. Methods for the measurement of CO$_2$ exchange are usually adapted to a neutral pH and frequently are indirect. It was therefore necessary to employ methods adapted to the acid range suitable for *Thiobacillus thiooxidans* which would allow the direct measurement of both CO$_2$ and O$_2$ since it was not possible to assume that the respiration (or other functions of metabolism) would be identical in the presence or absence of CO$_2$. Indeed, it was found that they were not. The methods employed use Dixon-Keilin flasks and are based upon the addition of from 30 to 600 µl. of CO$_2$ to a closed and equilibrated system, followed after a period, by the absorption of the remaining CO$_2$ by KOH. The addition of CO$_2$ has to be made rapidly so that its amount can be determined by the pressure change, before it becomes absorbed by the organism or partially dissolved in the medium. Two methods were used for CO$_2$ addition. (1) A small glass tube containing a small strip of filter paper saturated with Na$_2$CO$_3$ and dried was attached to the gas vent of a Dixon-Keilin flask with sealing wax. This was inserted into the side arm of the flask while the wax was still warm and pliable. In the side arm a solution of an inhibitor (usually 0.1 per cent HgCl$_2$ in 10 N H$_2$SO$_4$) in

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acid was placed. The whole system was equilibrated and when the CO₂ was to be added, the vent was turned slightly which dislodged the sodium carbonate tube, allowing it to drop into the sulfuric acid and liberate its CO₂. This insures the addition of pure CO₂. (2) The second method of CO₂ addition is as follows: The gas vents of the Dixon-Keilin flasks were connected with pressure tubing to a gas reservoir containing pure CO₂. The CO₂ was flushed through the vents until the system could be considered to contain pure CO₂. The vents were then plugged into the side arms. This means of filling the vents did not always yield pure CO₂ but blanks could be used to determine the actual amount of CO₂ added to each flask. Pure CO₂ could be insured, however, by evacuating the vents after they were plugged in the side arms and refilling with pure CO₂. Several evacuations and refills are necessary. The CO₂ is kept at a constant pressure in the reservoir (slightly higher than atmospheric pressure) by means of a water column and trap. Pressure tubing is used throughout to prevent the escape of CO₂ through the rubber and to permit evacuation of the vents. When CO₂ is to be added the vents are opened to the inside of the flask so that the CO₂ enters.

The amount of CO₂ added to the flasks by either method is easily calculated since during the short time the CO₂ remains in the side arm no appreciable quantity dissolves in the solutions of the flask or is taken up by the organism. The amount of CO₂ added (a) is calculated from the equation:

\[ a = V_{\alpha} \frac{T}{P_0} h_a \]  

(1) (Symbols those of Dixon (1934)).

in which \( h_a \) is the increase in pressure read on the manometer immediately after the addition of CO₂. After the allotted time has expired the sulfuric solution containing the inhibitor (which is used in both methods) is tipped into the flask from the side arm. This stops further cell activities and also releases any possible bound CO₂. After this addition it is necessary to allow a little time for equilibration since the addition of sulfuric acid results in the formation of some heat (heat of dilution) and a slight contraction of the mixture (which is very close to the contraction observed when sulfuric acid is mixed with water). When equilibrium has been reached, the KOH is added to the center cup by turning the bottom stopcock of the Dixon-Keilin flask. The resultant change in pressure (\( h_b \)) is read on the manometer after all the CO₂ from the gas phase has been absorbed (which takes about an hour) and enables one to calculate the CO₂ remaining in the atmosphere of the flask (b) since

\[ b = V_{\alpha} \frac{T}{P_0} h_b \]  

(2)

The difference \( a - b \) is the amount of CO₂ which has disappeared from the gas phase. The portion of this which has dissolved in the medium does not enter into the reading \( h_b \). It will, of course, eventually distill over into the KOH but the pressure change is caused only by the CO₂ absorbed from the gas phase.

The amount of oxygen taken up can be determined by comparing the reading just before CO₂ was added and after the CO₂ has been removed. This value is corrected for the contraction which occurs on mixing the sulfuric acid with water and which
is measured in the blank flask. This gives a measure of the oxygen uptake which is independent of the CO₂ measurement. It has the limitation, however, that only the initial and end values of oxygen uptake are determined. Intermediate points may be obtained by the use of a series of replicate flasks stopped at different intervals.

The main problem is then to discriminate between the CO₂ dissolved in the medium and that taken up by the cells. This was accomplished by running blank samples containing killed cells. Several methods of obtaining blank samples (as closely alike in composition to the living cell suspensions as possible) were employed. The principal methods employed were (1) heating of the suspension followed by cooling and shaking in air to equilibrate to atmospheric pCO₂ or (2) additions of HgCl₂ (0.1 per cent) or iodoacetate (0.001 per cent) in sulfuric acid. The addition of these materials (or the heat treatment) removes the CO₂ uptake due to living cells and leaves only that which dissolves in the medium or the cell substance. The observed CO₂ uptake under these conditions is quantitatively the same with all methods of killing (or in the entire absence of cells) and hence is only due to CO₂ solubility. This amount of dissolved CO₂ could be correlated with calculations based on the determined α value and the concentrations of CO₂ in the atmosphere.

This type of calculation can be made as follows: If α represents the number of milliliters of the gas dissolved in 1 ml. of the solution at a pressure of 1 atm., the amount of CO₂ dissolved at 1/n atmospheres will be 1/n.α (Henry's law). At the point of equilibrium, the amount of CO₂ dissolved in the solutions is equal to the amount soluble at the partial pressure of CO₂ in the atmosphere at that point, or if a is the amount of CO₂ added and x is the amount dissolved, it can be easily shown that:

\[ x = \frac{aV_F}{V_0 + aV_F} a \]

(3)

The α values for each medium can be determined with this formula from the blank containing killed cells. The volume of CO₂ dissolved in the fluid can thus be readily calculated for the exact experimental conditions employed and the CO₂ fixed by the cells can be determined by correcting the total CO₂ uptake observed by the amounts of CO₂ dissolved.

From the same blank the concentration of CO₂ in the laboratory atmosphere may be determined, from the difference between the reading before the CO₂ was added and the reading after all the CO₂ had been absorbed by KOH. The CO₂ concentration in the laboratory air varied from 0.04 to 0.07 per cent (corresponding to from 4 to 21 μl. CO₂ per flask). This is constantly recovered in blanks and is indicative of the accuracy of the method.

In calculating the amounts of CO₂ dissolved in the medium during the course of an experiment from the amounts of CO₂ present in the atmosphere of a flask at a given interval, equation (3) is slightly modified to:

\[ x = \frac{\frac{aV_F}{V_0 + aV_F}}{\frac{1}{V_0 + aV_F} a} \]

(4)

\[ 1 - \frac{aV_F}{V_0 + aV_F} a \]
in which \( a_t \) represents the amounts of CO\(_2\) left in the atmosphere of the flask at time \( t \), as calculated from the total amount present at the initial point less the total uptake plus the oxygen uptake over the time \( t \). See Table II for such a calculation.

The addition of the necessary amounts of gases sometimes extends the pressure changes beyond those measurable directly on the manometer. A method to extend the manometer range is therefore useful. None seems to have been described in the literature. Such a method, however, is easily derived from the equality of:

\[
VP = RT \quad \text{or} \quad V_1P_1 = V_2P_2 \quad \text{at constant} \quad T.
\]

When the volume of gas \( V_0 \) is increased \( x \) by changing the level of the manometer fluid in the closed arm over a height of \( e \) cm., the corresponding pressure \( P_0 \) will be decreased by \( y \) so that:

\[
(V_0 + x)(P_0 - y) = V_0P_0 \quad \text{or} \quad V_0y = x(P_0 - y)
\]

Similarly, if the fluid in the closed arm be lowered \( f \) centimeters the corresponding volume change \( z \) will cause a decrease \( y' \) so that

\[
V_0y' = z(P_0 - y').
\]

Thus:

\[
y/y' = \frac{x(P_0 - y)}{z(P_0 - y')} = \frac{e(P_0 - y)}{f(P_0 - y')}
\]

since \( x = r^2e \) and \( z = r^2f \) where \( r \) is radius of capillary tube of the manometer. But since \( P_0 \) is large (10,000 mm. of Brodie's solution) and \( y \) or \( y' \) are small, an accuracy of 1 per cent is possible if \( y \) and \( y' \) are not greater than 100 mm. (10 cm.) by considering \( P_0 - y = P_0 - y' \), from which:

\[
y/y' = 1/f.
\]

But:

\[
y = y' - w
\]

where \( w \) is the difference between the readings in the open arm of the manometer at \( e \) and at \( f \) or, therefore,

\[
y = \frac{we}{f - e}.
\]

If \( f \) be chosen to equal \( 2e \), then \( y = w \).

**CO\(_2\) Uptake in the Absence of Sulfur**

Carbon dioxide uptake during the oxidation of sulfur is an exceedingly complex phenomenon so that it is perhaps better to begin with the CO\(_2\) uptake by cells in the absence of sulfur. This was not expected inasmuch as the literature implies that CO\(_2\) uptake is limited to the period of sulfur oxidation. In fact, so rigidly has CO\(_2\) uptake been associated with growth of *Thiobacillus*
*thiooxidans* that, at least from evidence available until now, its uptake might be "growth bound." It is evident from the following sections in which CO₂ uptake is observed in resting cells, that the CO₂ fixation reaction is not "growth bound" but can occur in the absence of growth. CO₂ uptake in the absence of sulfur was further surprising in that Vogler (1942) had shown that a characteristic of endogenous respiration was the production of CO₂. This phenomenon (CO₂ liberation during endogenous respiration) was observed in suspensions from 14 day old cultures. In suspensions obtained from young cultures (about 7 days old), the endogenous oxygen uptake was much lower and CO₂ was often not liberated. Instead, these suspensions were capable of taking up small but definite amounts of CO₂ from the atmosphere. After CO₂ had been taken up to saturation the suspensions again formed CO₂ during endogenous respiration. After some study it became apparent that we were dealing with two types of suspensions with properties about as follows:

"Young suspensions"—harvested 6–8 days after inoculation
- Endogenous respiration low: Q₀₂(Ν) = 4–10
- Sulfur oxidation high: Q₀₂(Ν) = 2–3000
- CO₂ taken up during early stages of endogenous respiration.

"Old suspensions"—harvested after 12–14 days.
- Endogenous respiration high: Q₀₂(Ν) = 20–40
- Sulfur oxidation low: Q₀₂(Ν) = 2–400
- CO₂ released during endogenous respiration.

Suspensions of either type may be held for several weeks at refrigerator temperatures, but during this time they show some loss in activity. The "young" type of suspension may be converted into the older type by other means than aging; i.e., any method which causes the release of its energy stores. The old type of suspension may be converted into the young type by allowing it to oxidize sulfur for a short while. Only the young type of cell shows CO₂ fixation in the absence of sulfur.

The data contained in Table I show CO₂ fixation by young suspensions harvested after 7 days. This CO₂ fixation is observed in the absence of sulfur. Considering first the blank containing cells killed with HgCl₂ (flask 1), 680 µl. of CO₂ were added at zero time. Of this amount, 52 µl. dissolved in the solution. At the end of 15 hours the addition of KOH absorbed 656 µl. This amount plus the 52 µl. dissolved yields 708 µl. which is a recovery of the CO₂ added plus the CO₂ which was present in the air at the start of the experiment. At the end of 15 hours the addition of KOH absorbed 656 µl. This amount plus the 52 µl. dissolved yields 708 µl. which is a recovery of the CO₂ added plus the CO₂ which was present in the air at the start of the experiment. This amount of CO₂ originally present in the atmosphere of the flask is thus calculated as 708 — 680 = 28 µl. which corresponds to a concentration of CO₂ in the atmosphere of 0.06 per cent (about that usually found in the laboratory air). From this value the amount of CO₂ present in the other flasks may be calculated (Table I, column 2). Under these circumstances there was, of
course, a small amount of CO₂ dissolved in the solution at the start of the experiment, but this amount was very small and does not exceed 4 μl. so that it can be neglected. In the case of flasks Nos. 4 and 5, containing living cells, 573 and 654 μl. of CO₂ were added respectively. At the end of 15 hours of respiration the addition of KOH caused an almost negligible absorption (No. 4 = 27 μl.; No. 5 = 29 μl.) indicating that the CO₂ added had somehow disappeared. The organisms did not convert the CO₂ to bicarbonate ("bound" CO₂) since the addition of acid with the inhibitor from the side arm, to stop the activities of the cells before KOH was added, did not liberate any gas. Nor can solution in the medium account for the CO₂ removed since this uptake

<table>
<thead>
<tr>
<th>Flask contents</th>
<th>CO₂ in flasks Added at zero atmos. hr.</th>
<th>Total uptake CO₂ + O₂ Time in min.</th>
<th>Residual CO₂ at 15 hrs. absorbed in KOH</th>
<th>Total CO₂ taken up 15 hrs.</th>
<th>O₂ taken up 15 hrs.</th>
<th>CO₂ recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μl.</td>
<td>μl.</td>
<td>μl.</td>
<td>μl. per cent atm.</td>
<td>μl.</td>
<td>μl.</td>
</tr>
<tr>
<td>1. Blank, HgCl₂ killed cells</td>
<td>680</td>
<td>28</td>
<td>49</td>
<td>51 52 51 52 51</td>
<td>656</td>
<td>52</td>
</tr>
<tr>
<td>2. 2000 micrograms bacterial nitrogen</td>
<td>0</td>
<td>25</td>
<td>0</td>
<td>2 5 18 36 54</td>
<td>123</td>
<td>24</td>
</tr>
<tr>
<td>3. As 2</td>
<td>0</td>
<td>26</td>
<td>1</td>
<td>2 6 18 37 55</td>
<td>139</td>
<td>26</td>
</tr>
<tr>
<td>4. As 2</td>
<td>573</td>
<td>34</td>
<td>129</td>
<td>214 360 439 548 607</td>
<td>569</td>
<td>126</td>
</tr>
<tr>
<td>5. As 2</td>
<td>654</td>
<td>23</td>
<td>174</td>
<td>283 445 527 609 645</td>
<td>647</td>
<td>124</td>
</tr>
</tbody>
</table>

would have stopped at a level where an equilibrium between the amounts of CO₂ in the gas phase and the liquid phase was reached. In the case of cells treated in exactly the same way except killed with HgCl₂ (flask No. 1) this solubility amounted to only 52 μl. The data leave no doubt, therefore, that the CO₂ was actually fixed by the organisms since virtually all of the CO₂ disappeared from the atmosphere. The final amounts of CO₂ in the gas phase were found to be practically the same in all flasks, corresponding to a value close to the per cent of CO₂ in the laboratory air.

From the data of Table I, one can calculate the amount of CO₂ actually fixed at each interval measured, by correcting the total uptake observed in flasks 4 and 5 by the oxygen uptake observed in flasks 2 and 3 and for the CO₂ dissolved in the medium. This value can be calculated from the α value determined in flask 1 and the pCO₂ at each point. These values are given in
Table II which illustrates the method of calculation and the results are plotted in Fig. 1 which is more convenient for discussion.

### TABLE II

Calculations from the Data of Table I

For flask 5 only.

<table>
<thead>
<tr>
<th>Time, min.</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>15 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total uptake observed, μl</td>
<td>174</td>
<td>283</td>
<td>445</td>
<td>527</td>
<td>609</td>
<td>645</td>
<td>771</td>
</tr>
<tr>
<td>Oxygen uptake, μl</td>
<td>1</td>
<td>2</td>
<td>6</td>
<td>18</td>
<td>36</td>
<td>54</td>
<td>124</td>
</tr>
<tr>
<td>CO₂ uptake, μl</td>
<td>173</td>
<td>281</td>
<td>439</td>
<td>509</td>
<td>573</td>
<td>592</td>
<td>647</td>
</tr>
<tr>
<td>CO₂ left in atmosphere, μl</td>
<td>481</td>
<td>373</td>
<td>215</td>
<td>145</td>
<td>81</td>
<td>61</td>
<td>30</td>
</tr>
<tr>
<td>CO₂ dissolved, μl</td>
<td>52</td>
<td>40</td>
<td>23</td>
<td>16</td>
<td>9</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>CO₂ fixed by cells, μl</td>
<td>121</td>
<td>241</td>
<td>416</td>
<td>493</td>
<td>564</td>
<td>585</td>
<td>633</td>
</tr>
<tr>
<td>CO₂ fixed per 10 min. over interval, μl</td>
<td>186*</td>
<td>160</td>
<td>88</td>
<td>38</td>
<td>12</td>
<td>3</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* The time during which CO₂ was taken up in the first interval was but 6.5 minutes instead of 10. The lost 3.5 minutes represents the period over which CO₂ was added to the flask.

In Fig. 1, the fixation of CO₂ by the cells appears from the curve “CO₂ fixed.” It begins at a rapid rate which gradually decreases. During the first hour 78 per cent of all the CO₂ which was to be taken up by the cells had been fixed. The character of the CO₂ fixation is better studied from the line representing the rate of CO₂ fixation (in milliliters per 10 minutes over the interval recorded). If this curve is compared with the curve representing the amounts of CO₂ present in the flask at these intervals (which is proportional to the...
partial pressure of CO₂, it is apparent that they are almost parallel. This could be taken as evidence that the rate of CO₂ fixation is a function of the pCO₂. But if CO₂ fixation is a function of pCO₂, the nature of the fixation reaction is more clearly defined. It could be explained either by assuming that the CO₂ fixed is dissolved in the living cells (but not by the dead cells) in a manner expressed by the simple form of the Langmuir adsorption isotherm

\[ q = \frac{ap}{b + p} \]

where \( q \) is the amount of gas dissolved under a partial pressure \( p \) and \( a \) and \( b \) are constants, or by assuming that the fixation of CO₂ is a reversible enzymatic reaction. If CO₂ fixation be considered readily reversible and dependent upon pCO₂, it probably requires little energy.

Several experiments yielding entirely similar results to the one reported have been done, particularly in the study of the question of how much CO₂ could be taken up by cells under these conditions. This amount varies with the physiological condition of the culture but the maximum amount of CO₂ that can be taken up by cells in the absence of sulfur was found to be about 40 μl per 100 micrograms of bacterial nitrogen in suspensions which had recently been oxidizing sulfur. In the case of the experiment described in Table I, therefore, the maximum amount of CO₂ that could have been synthesized would be 800 μl., so that the 600–700 μl. added were less than the amount the cells could take up. This raises another problem. The suspension used in this experiment had been freshly harvested during which time it was exposed to CO₂ in the atmosphere. It could, therefore, take up CO₂ from its environment. On the other hand, the amount of CO₂ that can be taken up by such suspensions is limited. Why, then, are these suspensions able to take up more CO₂ when placed in Dixon-Keilin flasks?

It appears that the explanation may be found in the partial pressure of CO₂ in the atmosphere. It will be noted that the amount of CO₂ in the flasks at the end of their CO₂ uptake period was slightly higher than that of the laboratory air. After the partial pressure of CO₂ had been increased by the artificial addition of CO₂, this extra CO₂ was taken up by the cells until they had reached approximately the same equilibrium with respect to pCO₂ which had existed previously. The amounts of CO₂ added were not sufficient to saturate the organisms; if they had been, only a definite and constant amount of CO₂ would have been taken up. It therefore seems probable that within the limits represented by no CO₂ and saturation, the CO₂ is taken up by a reaction which reaches the equilibrium with the pCO₂ of the environment in which the cells have been grown.

One further point of interest in the data of Table I, is the absence of appreciable CO₂ liberation during the respiration in flasks 2 and 3. The amount of CO₂ recovered from the atmosphere at the end of the respiration was very close to that originally present in the laboratory air, hence the oxygen uptakes
recorded are actually oxygen uptakes in spite of the fact that no KOH was present during this period.

**CO₂ Fixation under Anaerobic Conditions**

Since CO₂ fixation is not limited to the time during sulfur oxidation, but occurs in the absence of oxidizable sulfur, it was of interest to determine whether CO₂ fixation would occur in the absence of oxygen. Data upon this problem are recorded in Table III. Here the same suspension was employed as in the experiment recorded in Table I. In flasks 3 and 4 a suspension of sulfur was placed in the side arm. The flasks were flushed with hydrogen until no oxygen was available, equilibrated, and the sulfur was added under hydrogen (so that no oxidation could occur). Known quantities of CO₂ were supplied, allowed to act for 2 hours, and then reabsorbed. The CO₂ actually fixed in the cells is essentially the same in the presence or absence of sulfur and can occur in the absence of oxygen.

After the residual CO₂ was absorbed, the flasks were flushed with CO₂-free air. After the oxygen uptake had continued for 2 hours the atmosphere was once more replaced with hydrogen and again the amount of CO₂ that could be fixed was determined. No attempt was made to measure the O₂ uptake during the 2 hour interval in which the gases were changed since the time necessary to change the atmosphere makes such measurements inaccurate. Instead, the values in Table III for oxygen uptake are based upon the values observed in other flasks of the same suspension, and hence only approximate the actual oxygen uptake during this interval. The actual oxygen uptake in the flasks was probably much less.

After the 2 hour respiration period, the flasks containing no sulfur did not regenerate the ability to take up CO₂ anaerobically, but the very small amount

<table>
<thead>
<tr>
<th>Flasks</th>
<th>5 ml. suspension</th>
<th>CO₂ uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Under H₂</td>
</tr>
<tr>
<td>1</td>
<td>Endogenous</td>
<td>75 μl.</td>
</tr>
<tr>
<td>2</td>
<td>Endogenous</td>
<td>71 μl.</td>
</tr>
<tr>
<td>3</td>
<td>+ sulfur</td>
<td>70 μl.</td>
</tr>
<tr>
<td>4</td>
<td>+ sulfur</td>
<td>74 μl.</td>
</tr>
</tbody>
</table>

The oxygen uptakes recorded are estimates based on values for endogenous respiration and S oxidation observed with the same suspension.
of O₂ uptake makes it impossible to be certain of this point. However, suspensions saturated with CO₂ in other instances were found to have lost the faculty of fixing any more CO₂ even after days of endogenous O₂ uptake so that it seems probable that endogenous respiration cannot regenerate the ability to fix CO₂. Indeed, such suspensions exhibit CO₂ liberation during the process of endogenous O₂ uptake, indicating a true endogenous breakdown of organic compounds.

Sulfur oxidation in flasks 3 and 4, however, restored the ability of the suspension to fix CO₂ at least partially. It may be noted that under the experimental conditions probably not more than half the cells were actually attached to the sulfur particles. These experiments have been repeated several times. From them one can only conclude that it is possible to oxidize sulfur in the absence of CO₂ and to store up the energy within the cell where it can later be used for CO₂ fixation under conditions during which sulfur oxidation is impossible.

In the data contained here, hydrogen was used as an “inert” gas. Hydrogen was in no case taken up during CO₂ fixation and thus could not have been active in any reaction. The CO₂ uptake under hydrogen was found to be the same as under nitrogen. The reduction of CO₂ by hydrogen which has been observed in other organisms thus does not seem to be important in Thiobacillus thiooxidans. Hydrogen was used more frequently than nitrogen in these experiments merely because we had a convenient source available, but the results were the same in pure nitrogen.

**CO₂ As an Oxidizing Agent**

In a previous paper (Vogler et al., 1942) evidence was given which indicates that the oxygen contained in the sulfate formed during sulfur oxidation might arise from the water in the medium rather than from the oxygen of the air. It therefore became of some interest to determine whether the CO₂ might not also act as an oxidizing agent. The CO₂ uptake during sulfur oxidation is a continuous process necessary for the supply of carbon to the cell. In this case the CO₂ uptake must be accounted for, either in terms of an increased level of cell oxidation or in the excretion of oxidized products. If one assumes that the overall oxidation level of the cell is about that of carbohydrate (0) and that the CO₂ is converted into cell material, it follows that for one molecule of CO₂ there will become available one molecule of O₂. If, then, sulfur oxidation be measured in the presence of CO₂, and if a molecule of oxygen be “liberated” for each CO₂ the Q₀₂(N) should decrease while the total uptake of O₂ + CO₂ should be relatively constant.

Data which show this actually to be the case are given in Table IV. During oxidation of sulfur the Q₀₂(N) is decreased by the presence of CO₂ (compare Q₀₂(N) of flasks 1 and 2 (no CO₂) with flasks 3-8 (with CO₂)), yet the sum of Q₀₂ + QCO₂ is about constant (column 3). This indicates that for each mole-
cule of CO₂ fixed, one less molecule of oxygen was taken up from the gas phase during sulfur oxidation.

It could not be determined whether the O₂ (which is thus apparently derived from CO₂) was set free into the medium or whether it was used directly in sulfur oxidation without such release. Since, however, during endogenous respiration we can find no O₂ liberated during CO₂ fixation and since under hydrogen or nitrogen no O₂ is formed in the presence or absence of sulfur, it may be assumed that the actual liberation of O₂ into the medium or the gas phase does not occur.

TABLE IV

<table>
<thead>
<tr>
<th>Flasks</th>
<th>Q₂</th>
<th>Q₃CO₂</th>
<th>Q₃O₂ + Q₃CO₂</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1580</td>
<td>0</td>
<td>1580</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>1600</td>
<td>0</td>
<td>1600</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>1020</td>
<td>710</td>
<td>1730</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>1490</td>
<td>283</td>
<td>1773</td>
<td>¥</td>
</tr>
<tr>
<td>5</td>
<td>1270</td>
<td>320</td>
<td>1590</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>1140</td>
<td>540</td>
<td>1680</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>1220</td>
<td>420</td>
<td>1640</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>1100</td>
<td>570</td>
<td>1670</td>
<td>2</td>
</tr>
</tbody>
</table>

Flasks 1 and 2 had no CO₂ added to the atmosphere. Flasks 1, 3, and 4 contained freshly harvested suspension. Flasks 2, 5, 6, 7, and 8 contained the same suspension aerated for 12 hours.

CO₂ Uptake during Sulfur Oxidation

The course of CO₂ fixation during sulfur oxidation is exceedingly complex. The previous sections have shown that the CO₂ may be fixed by the cells without sulfur; that, in the absence of oxygen, the sulfur does not aid CO₂ uptake; and that the presence of CO₂ may cause a lowering of the oxygen uptake. Yet the anaerobic experiments have shown that the energy of sulfur oxidation may be stored in the cell for a time, and used at a later time to fix CO₂. Thus the presence of CO₂ in the atmosphere results in CO₂ fixation which would deplete this stored energy supply. One might expect, therefore, that CO₂ would accelerate the overall rate of sulfur oxidation.

These changes are not always apparent in a single determination since they tend to compensate for one another. That they do occur, however, is apparent from the data plotted in Fig. 2. The points on these curves were obtained with a 10 ml. fresh bacterial suspension containing 200 micrograms bacterial nitrogen. The same suspension was used in parallel flasks for the determination of the other points recorded. The total uptake observed during
sulfur oxidation (which is due to both CO\(_2\) and O\(_2\) uptake) is plotted in curve 1. The oxygen uptake in the absence of CO\(_2\) was obtained from a parallel series of flasks containing KOH (curve 2). The CO\(_2\) uptake at intervals in a parallel series of flasks (curve 3) was obtained by the methods described previously. It has been corrected for CO\(_2\) solubility and represents only CO\(_2\) fixation. The CO\(_2\) uptake by this suspension shows a rapid onset which, after about half an hour settles to an even rate of CO\(_2\) fixation. This is the result of two processes: (1) the endogenous CO\(_2\) fixation (which has been drawn on the basis of the value determined at 2 hours and the data of Fig. 1 (shown in Fig. 2 as curve 4)), and (2) the CO\(_2\) fixation due to sulfur oxidation (which has been drawn in as curve 5 by subtracting curve 4 from curve 3). From the observed CO\(_2\) uptake (curve 3) and the rate of O\(_2\) uptake in parallel flasks without CO\(_2\) (curve 2) the total uptake of CO\(_2\) and O\(_2\) which should be observed in the flask containing CO\(_2\) can be calculated (curve 6). Comparison of this curve (No. 6) with the curve representing the actually observed uptake (No. 1) shows that during the first hour the observed uptake exceeds the calculated total uptake, which indicates a marked stimulation of sulfur oxidation by the presence of CO\(_2\). During the second hour the situation is reversed which indicates a depression in the oxygen taken out of the gas phase by the presence of CO\(_2\). This probably means a utilization of the oxygen of CO\(_2\) for sulfur oxidation.

Fig. 2. The course of CO\(_2\) fixation during sulfur oxidation by *Thiobacillus thiooxidans*. See text.
One may therefore conclude that in the early periods of sulfur oxidation the CO₂ is not utilized as an oxidizing agent but stimulates the rate of sulfur oxidation. After saturation of the endogenous capacity of CO₂ fixation, the oxidizing character of CO₂, resulting in a lower O₂ uptake, obscures this stimulating effect.

**Inhibition of CO₂ Fixation**

Four inhibitors were tested at concentrations which inhibit sulfur oxidation (Vogler et al., 1942) to determine their effect on CO₂ fixation.

The procedure consisted of a determination of the Qₒₒ(N) on sulfur of a dilute suspension containing 20 micrograms bacterial nitrogen per ml. This was determined over a period of 1 hour. The inhibitor was then added from the side arm to reach a final concentration indicated in Table V. The inhibited rate of sulfur oxidation was measured. CO₂ was then added. The extra uptake observed in the presence of the CO₂, less the oxygen taken up in parallel flasks containing no CO₂ was taken as a rough measure of the CO₂ fixed.

As appears from Table V, the inhibition of sulfur oxidation by pyruvate and sodium iodoacetate inhibits CO₂ uptake completely. The inhibition by iodoacetate in this case is more extensive on CO₂ fixation than on sulfur oxidation. Sodium azide and sodium arsenite which inhibit sulfur oxidation did not inhibit CO₂ uptake. However, the CO₂ uptake under these conditions must be considered as equivalent to the endogenous CO₂ fixation. Inhibition of sulfur oxidation must in any case eventually inhibit CO₂ fixation when the energy supplies of the cells have been depleted and the only energy for CO₂ fixation is that coming directly from sulfur oxidation.

Effects similar to that of pyruvate seem to be exerted on sulfur oxidation by lactic, succinic, and fumaric acids. Citric acid occasionally has a similar but smaller effect; malic acid has little or no influence. The apparent influence of these organic acids on sulfur oxidation (which has not been found with any other organic substrate) serves as an indication that they are somehow involved.

**TABLE V**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O₂ uptake</td>
</tr>
<tr>
<td>Na azide 1/100</td>
<td>100</td>
</tr>
<tr>
<td>Na arsenite 1/100</td>
<td>90</td>
</tr>
<tr>
<td>Na iodoacetate 1/10,000</td>
<td>10</td>
</tr>
<tr>
<td>Na pyruvate 1/150</td>
<td>100</td>
</tr>
</tbody>
</table>
in the processes of sulfur oxidation and CO$_2$ fixation. Further work is in progress on these materials. The indication is the more interesting since CO$_2$ fixation in heterotrophic organisms is known to be related to the action of some of these same compounds.

**DISCUSSION**

The studies described in the previous pages have defined, to some extent, the character of the CO$_2$ fixation process in the autotrophic bacterium. Of considerable interest is the demonstration that it is possible to oxidize the sulfur in the absence of CO$_2$ and to store the energy thus obtained within the cell where it can later be used for CO$_2$ fixation under conditions which do not permit sulfur oxidation. It is thus possible to separate the process of energy absorption (sulfur oxidation) from energy release (CO$_2$ fixation) and to study each independently.

The chemosynthetic process has been thought to be related to the photosynthetic process. The data obtained with the autotroph should now enable one to make some estimate of the extent of this relationship. However, according to the concepts developed by students of photosynthesis, the energy obtained from light is directly transmitted from the activated chlorophyll to an intermediate in the conversion of CO$_2$ to carbohydrate. These concepts leave no room for the storage of radiant energy within the cell in some form that can later be released for CO$_2$ fixation. Upon this basis, therefore, the chemosynthetic and photosynthetic processes are distinctly different in that in the former such a storage of energy occurs while in the latter no such storage is possible. On the other hand, there seem to be no experiments which have directly tested this hypothesis in photosynthesis; apparently the very direct connection between light energy and CO$_2$ fixation has almost always been gratuitously assumed. The available data on photosynthesis are not sufficient to exclude the possibility that the CO$_2$ fixation process in the autotrophic bacteria is directly related. Therefore the data obtained with the autotrophic bacteria raise the definite question whose answer does not appear to be already available and whose study may open new paths towards an understanding of photosynthesis, namely, is it possible to irradiate photosynthetic organisms in the absence of CO$_2$ and to store at least a portion of the radiant energy within the cell in a form which can later be used for CO$_2$ fixation in the dark?

**SUMMARY**

In a study of chemosynthesis (the fixation of CO$_2$ by autotrophic bacteria in the dark) in *Thiobacillus thiooxidans*, the data obtained support the following conclusions:

1. CO$_2$ can be fixed by “resting cells” of *Thiobacillus thiooxidans*; the fixation is not “growth bound.”
2. The physiological condition of the cell is of considerable importance in determining CO₂ fixation.

3. CO₂ fixation can occur in the absence of oxidizable sulfur in “young” cells. The extent of this fixation appears to be dependent upon the pCO₂.

4. CO₂ fixation can also occur under anaerobic conditions and the presence of sulfur does not influence such fixation.

5. However, in the CO₂ fixation by cells in the absence of sulfur, only a limited amount of CO₂ can be fixed. This amount is approximately 40 μl. CO₂ per 100 micrograms bacterial nitrogen. After a culture has utilized this amount of CO₂ it no longer has the ability to fix CO₂ but releases it during its respiration.

6. Relatively short periods of sulfur oxidation can restore the ability of cells to fix CO₂ under conditions where sulfur oxidation is prevented.

7. It is possible to oxidize sulfur in the absence of CO₂ and to store the energy thus formed within the cell. It is then possible to use this energy at a later time for the fixation of CO₂ in the entire absence of sulfur oxidation.

8. Cultures of *Thiobacillus thiooxidans* respiring on sulfur utilize CO₂ in a reaction which proceeds to a zero concentration of CO₂ in the atmosphere.

9. CO₂ may act as an oxidizing agent for sulfur.

10. Hydrogen is not utilized by the organism.

11. It is possible to selectively inhibit sulfur oxidation and CO₂ fixation.

BIBLIOGRAPHY

