

Keto Acids as Growth-limiting Factors in Autotrophic Growth of *Thiobacillus thiooxidans*

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ABSTRACT

When the strictly autotrophic bacterium *Thiobacillus thiooxidans* was grown on sulfur, keto acids accumulated in the medium until they reached an inhibitory level at which growth ceased. Much greater growth was possible if these substances were continually dialyzed out of the medium.

Thiobacillus thiooxidans is an autotrophic organism capable of growing, in an acid environment, entirely on mineral salts and elemental sulfur with CO₂ as its carbon source. The amount of growth under these circumstances is relatively small, and an increase in sulfur or in pCO₂ or neutralization of the acid formed not only does not stimulate further growth but is usually inhibitory. It has been shown that this organism excretes organic materials into the medium (7). These consist of a phospholipid related to phosphatidylinositol, which serves as a wetting agent for the sulfur (6, 7), and keto acids (D. S. Herson, M.S. Thesis, Rutgers, The State University, New Brunswick, N. J., 1965), the most prominent of which is pyruvic acid. This substance has also been shown to be excreted by the closely related autotrophic organism *Ferrobacillus ferrooxidans* (8).

It has also been demonstrated that it is possible to grow this organism on glucose, providing that a toxic material produced from the glucose was continuously removed (3). This material was identified as pyruvic acid. It therefore seemed possible that one of the factors which limited the growth of *T. thiooxidans* on sulfur might be the organic materials excreted into the medium, rather than the accumulation of acid as had been usually supposed. This paper demonstrates that markedly increased yield can be obtained if such organic materials are continuously removed and that the level of pyruvic acid found when the culture ceases to grow is close to that which prevents or inhibits growth when added externally. In addition, there appears to be an oxalacetic acid complex excreted into the medium which later is either reabsorbed by the cells or is otherwise altered. The role of this material is not clear. Pyruvate even at inhibitory levels is metabolized

by the cells in both growing cultures and cell suspensions, but the reason for its toxicity is not known.

MATERIALS AND METHODS

Culture. The Waksman strain of *T. thiooxidans*, originally obtained from R. L. Starkey, was employed.

Medium. The stock culture was maintained by weekly subculture in a basal salts medium previously described (3). The basal inorganic medium was dispensed in 100-ml quantities into 250-ml Erlenmeyer flasks and was autoclaved. Sulfur, previously sterilized by intermittent steaming, was added aseptically at 1.0 g per flask. Solutions of keto acids were sterilized by passage through a sterile membrane filter (Millipore Filter Corp., Bedford, Mass.) and were added as required in the experiments.

The flasks were inoculated with 1.0 ml of an 8-day-old culture of *T. thiooxidans*. This inoculum was approximately equivalent to 0.2 mg of protein. Flasks were incubated statically at room temperature (22 to 23 C) for 3 days. After the 3-day stationary period, the flasks were placed on a rotary shaker at 200 rev/min for the duration of the experiment. This type of procedure promotes contact between the bacteria and sulfur (4).

The flow-through system was that previously employed (3). Briefly, it consisted of a shaking flask containing a dialyzing sac through which fresh medium (free from sulfur) is passed; the sulfur is in the 100 ml of medium contained in the flask outside of the dialyzing tubing.

Keto acids were determined by the Frideman and Haugen (5) method with toluene as the extractant for pyruvate followed by ethyl acetate as the extractant for oxalacetate, both acids being identified by paper and thin-layer chromatography by use of tertiary amyl alcohol (5 parts), ethyl alcohol (1 part), and water (4 parts). Radioactive materials were sterilized by filtration through membrane filters (Millipore Filter Corp., Bedford, Mass.) and added aseptically. Counts were made in a Tracerlab Compumatic

counter and corrected for self-absorption. Other methods were those used earlier (3).

RESULTS AND DISCUSSION

The data of an experiment comparing the growth obtained on a flow-through with that on a shake-flask system are shown in Fig. 1. All flasks were provided with 1 g of sulfur, were inoculated with 1.08 mg of cell nitrogen from an 8-day-old culture of *T. thiooxidans*, and were held stationary for 4 days. Shaking was then started together with medium flow at a pH of either 5.2 or 1 in the appropriate flasks. The flow rate was 83 ml of fresh medium per hr through the dialyzing sac. It was evident that by continually washing out some toxic products in the flow-through cultures the yield was markedly increased. It appeared that acid may have exerted an inhibitory action (or enhanced the toxicity of the toxic agent), but, even at pH 1, there was a marked increase in growth in the flow-through system. The medium remaining in the flasks at the end of the experiment was analyzed for pyruvate. A level of 0.25 $\mu\text{g}/\text{ml}$ was found in the flow-through system (pH 5), whereas 5.1 $\mu\text{g}/\text{ml}$ was found in the shaken culture. The level which prevented growth when added to growing cultures was approximately 4 $\mu\text{g}/\text{ml}$. Thus, it appears that a limiting factor in the growth of this organism in shaken cultures was the accumulation of pyruvate in the medium.

To estimate the pyruvate, the media had always been concentrated in vacuo (below 60 C), but, since it was acid, there was the possibility that a more labile compound, such as oxalacetate, was present and had broken down to

pyruvate during the concentration procedure. This supposition was based upon reports (1, 2, 9, 10) that oxalacetic acid (and aspartic acid) were among the first products of CO_2 fixation by this organism. Dinitrophenylhydrazine was therefore added directly to the spent media from the shaken cultures [cells removed by membrane filtration (Millipore Filter Corp., Bedford, Mass.)], and this was then concentrated by removal of water in vacuo. The concentrated hydrazones were first extracted with toluene to remove the hydrazones of pyruvic acid and then were extracted with ethyl acetate to remove the remainder. The color found in the toluene (after reaction with strong alkali) was taken as a measure of pyruvate, and that found in the ethyl acetate was taken as a measure of oxalacetate (confirmed by paper and thin-layer chromatography). Both were found, and in the experiment shown in Fig. 1 (at pH 5.2), the flow-through system showed 0.8 $\mu\text{g}/\text{ml}$ of oxalacetate, whereas the stationary culture had 6.25 $\mu\text{g}/\text{ml}$.

The oxalacetate and pyruvate present during the course of growth in a shaken-flask culture were measured (Fig. 2). It is evident that, for the early part of the growth curve, the log phase of growth, there was an accumulation of oxalacetic acid in the medium. This reached a peak at just the point where log growth ceased (9 days). Subsequently, there was a decrease in oxalacetic acid but not a corresponding increase in pyruvic acid. The latter tended to increase gradually until it reached a level close to 6.5×10^{-5} M during the stationary phase. As shown earlier (3), the addition of 2×10^{-5} to 4×10^{-5} M pyruvate completely inhibits growth when added with the inoculum. However, more pyruvate than this is required when it is added after growth has begun. As such, then, the pyruvate present is sufficient

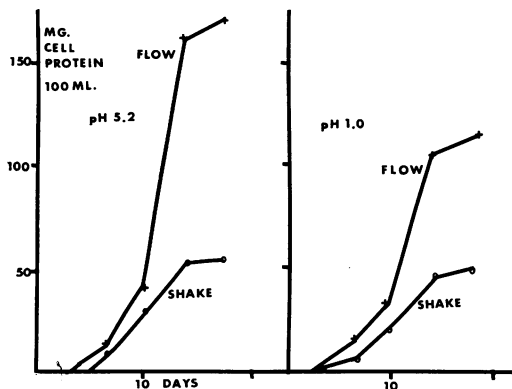


FIG. 1. Comparison of growth of *Thiobacillus thiooxidans* in flow-through cultures and in shaken cultures. Final pH (19 days), with media at an initial pH of 5.2, was 3.0 in the flow-through culture and was 0.85 in the shake culture. With media at an initial pH of 1.0, the final pH in both the flow-through and shake cultures was 0.80.

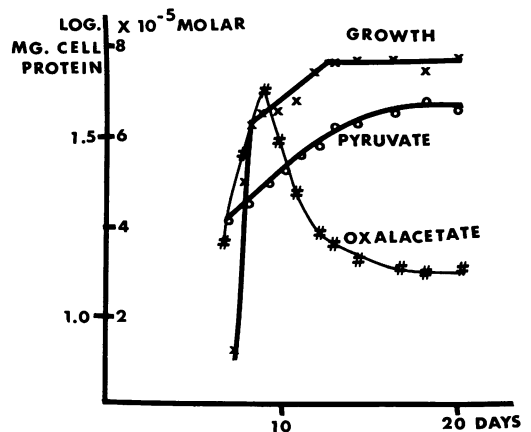


FIG. 2. Accumulation of oxalacetic acid and pyruvic acid during growth in shaken cultures.

to account for the lack of further growth in the culture.

If oxalacetate was added to the medium with the inoculum, its toxicity was in the same range as that of pyruvate, i.e., 2×10^{-5} to 4×10^{-5} M. The fact that over 6.7×10^{-5} M oxalacetic acid accumulates at the peak point (giving at 9 days a total keto acid level of 11.8×10^{-5} M) before evident limitation in growth occurs might be because the amount of keto acids required to inhibit growth is greater when the culture is growing vigorously than when the keto acid is supplied with the inoculum. However, the data found may also indicate that the material measured as oxalacetate is indeed a less toxic complex of it. The substance determined was, in fact, the dinitrophenyl hydrazone of oxalacetic acid, which might have resulted from the breakdown of a more complex substance or from reaction of this substance with dinitrophenyl hydrazine. We suggest this latter alternative, because we found that, under some circumstances, when the spent medium is concentrated without addition of dinitrophenyl hydrazine, a spot appears on chromatograms in methanol-chloroform (1:4) on silica gel paper; the spot moves more rapidly than pyruvate or oxalacetate and, by spray reagents, reacts with dinitrophenyl hydrazine (indicating a keto group), rhodamine S (indicating lipid), and phosphate, the presence of which suggests an oxalacetic acid-phospholipid complex. However, characterization of this complex is not clearly established, and much more work is required. There are in the medium other phospholipids, particularly the one related to phosphatidyl inositol. Their influence on the movement of keto acids or the association between these lipids and keto acids during the concentrations of the media results in a complex analytical situation which has not yet been resolved.

Addition of pyruvate and oxalacetate inhibited growth on sulfur at a concentration of 2×10^{-5} M. Comparable inhibition was shown by malonic, itaconic, dihydroxifumaric, and hydroxypyruvic acids. It requires roughly 10 times as much (i.e., 10^{-4} M) citric, *trans*-aconitic, isocitric, α -ketoglutaric, succinic, and malic acids for comparable inhibition.

The cause of the toxicity is not clear. In resting cells, 2×10^{-4} M pyruvate completely inhibited sulfur oxidation, but 10^{-4} M may have been slightly stimulating and 10^{-5} M consistently showed an increased oxygen uptake with stimulations varying from 30 to 100%. Analysis of the residual pyruvate (possible with decreasing accuracy down to 10^{-4}) showed that pyruvate decreased in the fluid surrounding the cells even at 10^{-3} M where sulfur oxidation was inhibited.

Radioactive pyruvate determinations indicate

that much of the pyruvate utilized from the surrounding medium may be associated with the cells as pyruvate, although some (on the order of 5 to 10%) seems to be converted into materials not extractable with trichloroacetic acid.

Studies with radioactive pyruvate and oxalacetate have so far contributed little to the solution of the problem of pyruvate toxicity; these merely demonstrate that at 10^{-4} M both carbonyl-labeled and carboxy-labeled pyruvate are slowly converted to CO_2 and to cell substance, but the total amount converted, even over periods as long as 24 hr, is only about 20% of that available. However, what is clear from the available data is that it is the accumulation of inhibitory organic materials in the medium which limits the growth of this autotroph and also very probably the activity of the cell suspensions obtained from such cultures.

ACKNOWLEDGMENT

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