

## Yield Coefficients of *Thiobacillus neapolitanus* in Continuous Culture

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*Thiobacillus neapolitanus*, when grown in continuous culture with thiosulfate limiting growth, possessed an apparent maximal molar growth yield of 8.0 g (dry weight) per mole of thiosulfate. The substrate requirement for energy of maintenance was the highest yet reported, amounting to 21.8 mmoles of thiosulfate per g per hr. The molar growth yield, corrected for this maintenance energy requirement, was 13.9 g (dry weight) per mole of thiosulfate. It was concluded that substrate-level phosphorylation during sulfite oxidation accounted for about 45% of the adenosine triphosphate (ATP) requirement for CO<sub>2</sub> assimilation and maintenance during growth on limiting thiosulfate, that three sites of energy conservation exist in the electron-transport chain terminating in oxygen, and that 7.8 moles of ATP are required to fix and assimilate 1 mole of CO<sub>2</sub> into cell material.

The possibility that both substrate level and oxidative phosphorylation function as energy-conserving mechanisms during thiosulfate oxidation by the thiobacilli has been previously suggested (18, 19). Direct evidence for the occurrence of substrate-level phosphorylation in extracts of *Thiobacillus thiooparus* during the oxidation of sulfite was found by Peck (11), who subsequently questioned the role of oxidative phosphorylation in energy conservation by the thiobacilli (12). However, according to Kelly and Syrett (8), the uncoupler of oxidative phosphorylation, 2,4-dinitrophenol, inhibits CO<sub>2</sub> fixation by resting cells of *T. thiooparus* during both thiosulfate and sulfide oxidation, and a direct demonstration of oxidative phosphorylation by extracts of *T. neapolitanus* was made by Hempfling and Vishniac (6).

To estimate the relative roles of substrate-level and oxidative phosphorylation in energy conservation during growth on thiosulfate, the molar growth yield on thiosulfate as well as the energy of maintenance of *T. neapolitanus* have been estimated by the method of continuous culture in a constant pH chemostat. Trudinger (16) has cultivated *T. neapolitanus* continuously but has not reported the characteristics of steady-state growth.

### MATERIALS AND METHODS

*T. neapolitanus* [*Thiobacillus* X (10)] was kindly supplied by P. A. Trudinger, and was maintained in

the frozen state and by liquid transfer in the medium of Vishniac and Santer (18) containing 54 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> and 130 mM potassium phosphate buffer (pH 7.0).

A chemostat vessel of 1,100-ml culture volume was constructed by the Blaessig Glass Specialties Co., Inc., Rochester, N. Y. (6). Provision was made for the regulation of pH by the automatic addition of 1 or 2 M K<sub>2</sub>CO<sub>3</sub> (Beckman expanded scale pH meter, calomel and glass electrodes, JKM Stat relay, and a solenoid-operated magnet valve), of stirring rate (Bodine motor with rectifier unit and magnetic stirrer attachment), of aeration rate (Matheson flowmeter No. 603), of medium flow (Beckman solution pump), and of temperature (Yellow Springs Instrument Co. Pyrex thermistor probe, thermistor relay, and an infrared lamp). The level of medium in the culture vessel was maintained constant by providing an overflow portal. Air was sterilized by passage through a drying tube packed with sterile cotton.

Measurement of bacterial growth was made by determining the optical density of the effluent fluid at 660 m $\mu$  through a 1-cm light path with water as blank. To determine bacterial dry weight, cells were collected from 100-ml portions of the effluent medium by centrifugation in the cold and were resuspended in 0.1 volume of water. Triplicate samples of that suspension were dried to constant weight at 85 to 90 C. Since elemental sulfur was not formed, it did not interfere with turbidimetric or gravimetric measurements.

Concentrated sodium thiosulfate and potassium phosphate solutions were sterilized separately by filtration through a membrane filter (pore size, 0.45  $\mu$ ; Millipore Filter Corp., Bedford, Mass.) and were added after 15 liters of basal medium had been sterilized at 121 C for 60 to 75 min and allowed to cool to room temperature.

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Heterotrophic contamination was assessed by plating appropriate dilutions of the effluent medium on Difco Tryptic Soy Agar. Those cultures which showed more than 0.1% heterotrophic contamination were not used in the following calculations.

Thiosulfate was determined by the thiocyanate procedure (14), phosphate by the method of Fiske and SubbaRow (4), ammonium ion by the Nessler procedure, and magnesium ion by titration with standard ethylenediaminetetraacetate. All of these components were present in excess except thiosulfate.

## RESULTS

**Relationship of molar growth yield to dilution rate.** Figure 1 shows the steady-state optical density of the effluent medium and the apparent molar growth yield as a function of the dilution rate  $D$  when thiosulfate was made the limiting growth factor. [ $D = f/V$ , where  $f$  is the flow rate of fresh medium in milliliters per hour and  $V$  is the culture volume in milliliters (7).] The medium employed was that of Vishniac and Santer (18) containing 37 mM potassium phosphate and 52 mM  $\text{Na}_2\text{S}_2\text{O}_3$ . The pH was maintained at 6.7 to 6.9, and the temperature was  $30 \pm 0.2$  C. The rate of aeration was 3.8 to 4.0 liters per min. Beginning at a dilution rate of 0.081/hr, the cell population was permitted to reach a steady state, and measurements of optical density and dry weight of cells per unit volume were made for a period of two to three generations; then a small increase in dilution rate was made and the process was repeated. The maximal mean molar growth yield reached was 8.0 g (dry weight) per mole of thiosulfate oxidized to sulfate, obtained at a dilution rate just below the critical dilution rate  $D_c$ . [ $D_c$  is reached when  $D$  exceeds  $\mu_{\max}$ , where  $\mu_{\max}$  is the maximal specific growth rate (7).] This value was the average of three separate chemostat runs in which  $D$  was increased in a stepwise manner from

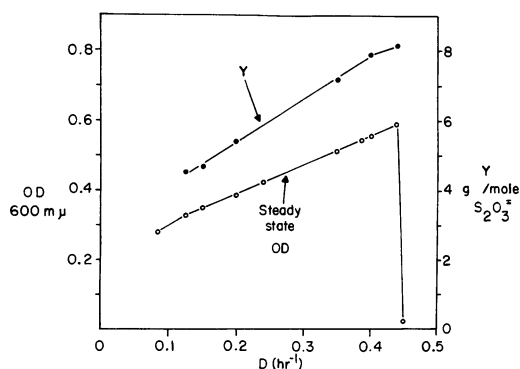


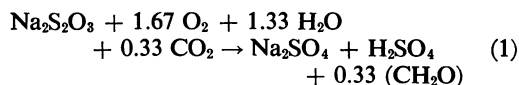
FIG. 1. Steady-state population density (optical density at 660  $m\mu$ ) and molar growth yield as a function of the dilution rate  $D$ .

$D = 0.08/\text{hr}$  to  $D_c$  (0.45/hr). Figure 1 was prepared from data obtained from one of these runs and is representative of all runs made.

The amount of alkali consumed per mole of thiosulfate added was two equivalents at all dilution rates, when corrected for the buffering capacity of the medium, indicating that thiosulfate was completely oxidized to sulfate. In agreement with this observation was the fact that only a small amount of thiosulfate could be detected in the effluent medium (0.1 mM thiosulfate at  $D = 0.1/\text{hr}$  and 0.9 mM thiosulfate at  $D = 0.40/\text{hr}$ ). Growth yield data are not corrected for the small amount of thiosulfate remaining in the spent culture medium.

It is obvious from Fig. 1 that the growth yield was dependent upon the dilution rate. The possibility that a strain more efficient in energy conservation was being selected as the dilution rate was progressively increased was eliminated by the observation that reduction of  $D$  after the steady state had been reached at  $D = 0.40/\text{hr}$  brought about a corresponding reduction in molar growth yield.

**Growth stoichiometry and energy of maintenance.** Assuming that 50% of the dry weight of *T. neapolitanus* is carbon, the oxidation of 1 mole of thiosulfate to 2 moles of sulfate brings about (at  $D = 0.42/\text{hr}$ ) the assimilation of 4.0 g or 0.33 g atom of C according to the reaction



At lower values of  $D$ , the equation of growth will obviously differ from equation 1.

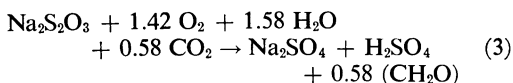
However, the dependence of molar growth yield on the dilution rate during growth in the chemostat suggests that the energy requirement for cell maintenance was high, leading to an underestimate of the true molar growth yield. Pirt's recent treatment of the phenomenon of energy of maintenance in continuous culture (13) has suggested that the molar growth yield, corrected for energy of maintenance ( $Y_G$ ), can be calculated from the relationship

$$1/Y = m/\mu + 1/Y_G \quad (2)$$

where  $m$  is the substrate requirement for energy of maintenance,  $\mu$  is the specific growth rate, and  $Y$  is the apparent molar growth yield. As shown in Fig. 2, a plot of  $1/Y$  versus  $1/D$  (remembering that in the steady state  $\mu = D$ ) resulted in a function whose intercept at the ordinate is the reciprocal of the molar growth yield on thiosulfate, corrected for energy of maintenance, and whose slope is  $m$ . From these considerations, it

is concluded that the molar growth yield of *T. neapolitanus* with respect to thiosulfate, corrected for energy of maintenance, is 13.9 g (dry weight) per mole of thiosulfate, and that the substrate requirement of energy of maintenance ( $m$ ) is 21.8 mmoles of thiosulfate per g (dry weight) per hr. The substrate requirement for energy of maintenance appears to be constant up to a generation time of 5 hr. At slower dilution rates,  $m$  is diminished to about 10.5 mmoles of thiosulfate per g (dry weight) per hr, and the molar growth yield, corrected for energy of maintenance, is 7.7 g (dry weight) per mole of thiosulfate.

If there were no substrate requirement for energy of maintenance (i.e.,  $m = 0$ ), the oxidation of 1 mole of thiosulfate would bring about the incorporation of 6.95 g or 0.58 g atom of C into cell material, and the equation of growth corrected for  $m$  would be written:



This equation describes the growth stoichiometry at higher dilution rates. However, at dilution rates corresponding to generation times of 5 hr or more, equation 1 approximates the equation of growth

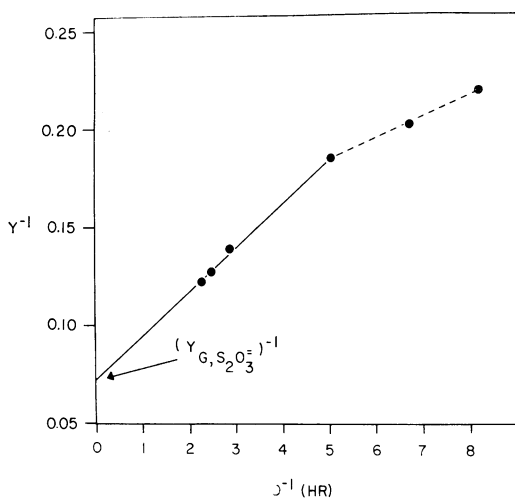


FIG. 2. Estimation of substrate requirement for energy of maintenance ( $m$ ) and molar growth yield corrected for  $m$ . Solid line shows data obtained at growth rates in excess of a generation time of 5 hr:  $m$  (slope) = 21.8 mmoles per g per hr; molar growth yield, corrected for energy of maintenance, = 13.9 g (dry weight) per mole of thiosulfate. Dashed line describes data obtained at growth rates slower than a generation time of 5 hr:  $m = 10.5$  mmoles per g per hr; molar growth yield, corrected for energy of maintenance, = 7.7 g (dry weight) per mole of thiosulfate.

corrected for substrate requirement for energy of maintenance. In the following discussion, conclusions will be based upon a growth stoichiometry given by equation 3, since this relationship appears to be optimal.

## DISCUSSION

*ATP requirement for CO<sub>2</sub> assimilation.* Two high-energy phosphate bonds are generated per mole of thiosulfate oxidized to sulfate by the substrate-level phosphorylation pathway of sulfite oxidation as proposed by Peck (11). [Sulfite oxidation does not appear to be linked to oxidative phosphorylation since extracts of *T. neapolitanus* oxidize sulfite without concomitant adenosine triphosphate (ATP) formation (6), 100-fold purified sulfite oxidase from *T. neapolitanus* is not cytochrome-linked (W. P. Hempfling, Ph.D. Thesis, Yale Univ., New Haven, Conn., 1964), the addition of sulfite to cell extracts does not reduce endogenous cytochromes or flavoprotein (W. P. Hempfling, unpublished data), and the addition of uncouplers of oxidative phosphorylation to sulfite-supplemented extracts does not stimulate the rate of sulfite oxidation (Hempfling, Ph.D. Thesis).] According to equation 3, 1 mole of thiosulfate is completely oxidized during the assimilation of 0.58 mole of CO<sub>2</sub> by growing *T. neapolitanus*. One mole of O<sub>2</sub> is required for terminal sulfite oxidation, yielding 2 moles of ATP by substrate-level phosphorylation. The remaining 0.42 mole of O<sub>2</sub> is therefore available for reduction through electron-transport reactions coupled to oxidative phosphorylation. If three sites of oxidative phosphorylation exist in the electron-transport chain, beginning with thiosulfate and terminating with oxygen, then the reduction of 0.42 mole of O<sub>2</sub> or 0.84 g atom of O yields  $0.84 \times 3 = 2.52$  moles of ATP. If two sites or one site is assumed, the ATP yield would be 1.68 or 0.84 moles of ATP, respectively.

The total contribution of substrate-level and oxidative phosphorylation to ATP production would be 2.84, 3.68, or 4.52 moles of ATP if one, two, or three sites of oxidative phosphorylation are assumed, respectively. This amount of energy must suffice to drive the conversion of 0.58 mole of CO<sub>2</sub> into cell material. Hence, to assimilate 1 mole of CO<sub>2</sub>, 4.9, 6.4, or 7.8 moles of ATP is required. To assimilate 1 mole of CO<sub>2</sub> via the Calvin-Benson pathway 3 moles of ATP is required per mole of CO<sub>2</sub>, as well as 2 moles of reduced nicotinamide adenine dinucleotide (NADH<sub>2</sub>). In *Thiobacillus*, these reducing equivalents probably arise through energy-dependent reversed electron transport; Aleem (1) concluded that 1 mole of ATP is sufficient to bring about the

reduction of one mole of NAD with reduced cytochrome *c* as reductant. Therefore, two additional moles of ATP is required to furnish reducing equivalents, bringing the expected requirement for carbohydrate synthesis from CO<sub>2</sub> to 5 moles of ATP per mole of CO<sub>2</sub> fixed.

Vishniac et al. (17) have presented calculations, based on the above considerations and the molar growth yield determinations of Bauchop and Elsdén (2), which indicate that about 2.9 moles of ATP per g atom of C is required to convert carbohydrate to cell material. The final predicted amount of ATP required to convert 1 mole of CO<sub>2</sub> to cell material is  $3 + 2 + 2.9 = 7.9$  moles of ATP. It is seen that this figure corresponds closely to the above estimate of 7.8 moles of ATP per mole of CO<sub>2</sub>, based on the assumption of three phosphorylation sites in the electron-transport chain of *T. neapolitanus*. Consequently, it is tentatively concluded that three sites of oxidative phosphorylation exist in *T. neapolitanus*.

The previous calculations of Elsdén (3), who suggested that 12 or more moles of ATP was required for the assimilation of 1 mole of CO<sub>2</sub>, were based on experiments with resting hydrogenomonads (9). It is probable that the uncoupling of CO<sub>2</sub> assimilation from respiration led to an overestimate of the ATP requirement.

*Electron transport and phosphorylation.* The absence of oxidative phosphorylation during the oxidation of sulfite requires that oxidative phosphorylation be associated only with the oxidation of the outer sulfur of thiosulfate to the level of sulfite. Electrons derived from this source may have one of two fates: they either pass to oxygen through the cytochrome chain, yielding three moles of ATP per pair of electrons, or they reduce NAD, thereby consuming one molecule of ATP per NAD reduced. The amount of cell mass formed is a measure of the energy-linked reduction of NAD; it is estimated from the data presented that about 58% of the electrons derived from the outer sulfur of thiosulfate are diverted to CO<sub>2</sub> fixation. Thus, only 4.52 moles of ATP (2.52 moles from oxidative and 2 from substrate-level phosphorylation) is formed per mole of thiosulfate during the oxidation of 1 mole of thiosulfate by growing cells, rather than the 8 moles of ATP expected. The sites of energy conservation in the terminal electron transport pathway are unknown, but the oxidation of NADH<sub>2</sub> by extracts of *T. neapolitanus* is not linked to oxidative phosphorylation (6).

Because of the relatively large consumption of oxygen through sulfite oxidation, substrate-level phosphorylation contributes much more to ATP production in *T. neapolitanus* than, for instance, in an aerobic heterotroph oxidizing glucose

through the Embden-Meyerhof-Parnas pathway and the Krebs tricarboxylic acid cycle, where substrate-level phosphorylation accounts only for about 15% of net energy conservation. Substrate-level phosphorylation in *T. neapolitanus* amounts to about 45% of net energy conservation. Thus, Peck's suggestion (12) that oxidative phosphorylation may contribute less than expected to energy conservation in the thiobacilli is qualitatively borne out in these studies.

*Significance of energy of maintenance.* The amount of substrate required for energy of maintenance during growth is much higher in *T. neapolitanus* than in those heterotrophs for which data are available (13). The meaning of this observation is not clear, especially since the role of "maintenance energy" is not known. It is possible that a large proportion of it might be utilized for active transport processes (5) or for osmotic work, or it might simply be lost through an "energy leak." At low growth rates, the substrate requirement for energy of maintenance is only half that at higher growth rates; the significance of this observation is unknown.

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