The Physiological Role of Tetrathionate Respiration in Growing *Citrobacter*

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SUMMARY

The reduction of tetrathionate to thiosulphate by anaerobically growing *Citrobacter* caused a significant rise in specific growth rate and molar growth yield. It enabled also anaerobic growth on non-fermentable carbon sources and resembled therefore the respiratory reduction of nitrate to nitrite. The efficiency of energy production by tetrathionate respiration amounted to only two-thirds that of nitrate respiration.

INTRODUCTION

Pollock and co-workers (Pollock, Knox & Gell, 1942; Knox, Gell & Pollock, 1943; Pollock & Knox, 1943) discovered that some facultative anaerobic bacteria reduce tetrathionate enzymatically according to the equation

Thiosulphate and H^+ accumulated in the medium. The reaction is catalysed by tetrathionate reductase, an inducible enzyme, whose synthesis and activity is inhibited in aerobiosis by oxygen (Pollock & Knox, 1943; Pichinoty & Bigliardi-Rouvier, 1963; Kaprálek & Pichinoty, 1970). Knox *et al.* (1943) concluded that this bacterial activity may be a form of anaerobic respiration analogous to the reduction of nitrate to nitrite.

Although much work has been done in studying the energy aspects of nitrate respiration (e.g. Forget & Pichinoty, 1964; Hadjipetrou & Stouthamer, 1965; Downey, 1966; Wimpenny & Cole, 1967) little attention has been paid to the energy or other physiological significance of tetrathionate reduction, in spite of the fact (LeMinor & Pichinoty, 1963; Papavassiliou, Samaraki-Lyberopoulou & Piperakis, 1969) that tetrathionate reduction is almost as widespread as nitrate reduction among the facultative anaerobes. Knox (1945) demonstrated that addition of tetrathionate (which is reduced to thiosulphate) prolonged the logarithmic phase of anaerobic growth of *Bacterium paratyphosum-B* in broth and increased the growth yield. In this respect the effect of tetrathionate resembled that of oxygen. On the other hand, de Groot & Stouthamer (1969) showed that electron transfer to tetrathionate in *Proteus mirabilis* did not have a significant influence on anaerobic growth. In this report, the effect of tetrathionate respiration on the growth rate and growth yield of *Citrobacter* is described.

METHODS

Organism and growth conditions. The original strain isolated by Pollock (1946) and labelled '1433', identified as *Citrobacter* (Sedlak & Slajsova, 1967), was grown at 30 °C on M₁ medium (Kaprálék & Pichinoty, 1970) in which lactose was replaced by glucose, galactose or pyruvate (2 g/l), sterilized separately. The pH was adjusted to 6.8 before sterilization for 20 min at 127 °C. Yeast extract, Casamino acids and bacto-peptone were omitted in the minimal medium

M₀, in which growth on lactate, glycerol and succinate (2 g/l) was tested. Anaerobic cultures were grown statically in 100 ml of the medium in a 150 ml cylinder placed in a water bath. Oxygen-free nitrogen was bubbled through the medium for 10 min before inoculation and passed over the surface of the culture during cultivation. If tetrathionate or nitrate served as final electron acceptors, a solution of K₂S₄O₆ or KNO₃ (sterilized by filtration) was added (2 g/l). Aerobic cultures were grown in 100 ml of the medium in a 1000 ml Erlenmeyer flask on a reciprocating shaker. A culture growing exponentially on the same medium under identical conditions served as inoculum. Growth was monitored by measuring the extinction at 420 nm on a Hilger-Spekker colorimeter. Extinction 1.0 was equivalent to 0.31 g bacterial dry wt/l of culture. The molar growth yield (*Y*) (g of bacterial dry wt formed/mol of substrate consumed) was determined during exponential growth; the increment of bacterial dry wt concentration (*x*) (μg/ml) was plotted against the increment of consumed substrate concentration (*c*) (μmol/ml). The slope of the resulting straight line is *Y* according to Monod's (1942) equation relating the growth rate dx/dt and the rate of substrate utilization $-dc/dt$

$$\frac{dx}{dt} = Y \frac{-dc}{dt},$$

from which

$$Y = \frac{dx}{-dc} \text{ (g/mol)}.$$

The specific metabolic rate of substrate utilization (*q*) (mol of substrate consumed/g of bacterial dry wt/h) was calculated from the following relationships (Pirt, 1957):

$$\frac{-dc}{dt} = qx, \quad \frac{dx}{dt} = \mu x,$$

where μ is the specific growth rate (h⁻¹). Dividing both equations we obtain the expression

$$\frac{dx}{-dc} = \frac{\mu}{q} = Y,$$

from which

$$q = \frac{\mu}{Y} \text{ (mol/g/h)}.$$

Assays. Glucose and galactose were determined by the method of Park & Johnson (Ashwell, 1957). The sensitivity of this method allowed dilution of the culture filtrate 1:1000 which eliminated the interference of tetrathionate and thiosulphate. Pyruvate was assayed by the method of Friedemann (1957) and nitrite by the method of Nicholas & Nason (1957). Thiosulphate was determined iodometrically (Pollock & Knox, 1943). The micro-organism used in our work did not reduce thiosulphate enzymatically and therefore the thiosulphate found in the medium corresponded quantitatively to the tetrathionate reduced.

RESULTS

The left part of Fig. 1 demonstrates the effect of different final electron acceptors on the growth rate of *Citrobacter* and the right part shows the time course of the reduction of tetrathionate and nitrate. Anaerobic respiration of tetrathionate resulted in the increase of specific growth rate on three different sources of energy and electrons (Table 1). The increase was approximately equal to that resulting from nitrate respiration but amounted to only about half of the increase brought about by aerobic respiration. The increase in growth rate caused by any of the three electron acceptors was highly significant on all three sources of energy and electrons.

Fig. 1. The growth of *Citrobacter* on galactose in M_1 medium with different exogenous electron acceptors. $\circ$, Anaerobically without acceptor; $\triangle$, anaerobically with $K_2S_4O_6$; $\square$, anaerobically with KNO_3 ; $\bullet$, aerobically; $\blacktriangle$, μ mole of reduced $K_2S_4O_6$ /ml; $\blacksquare$, μ mole of reduced KNO_3 /ml.

Table 1. The effect of different exogenous electron acceptors on the specific growth rate of *Citrobacter*

Cultures (100 ml) grown statically under N_2 or in shaken flasks in air at 30 °C.

Source of energy and electrons	Exogenous acceptor of electrons	Specific growth rate $\mu \pm s_x$ ($n = 3$) (h^{-1})
Glucose	None	0.51 ± 0.03
	$S_4O_6^{2-}$	0.82 ± 0.01
	NO_3^-	0.75 ± 0.01
	O_2	0.99 ± 0.01
Galactose	None	0.35 ± 0.01
	$S_4O_6^{2-}$	0.55 ± 0.01
	NO_3^-	0.55 ± 0.01
	O_2	0.77 ± 0.02
Pyruvate	None	0.42 ± 0.01
	$S_4O_6^{2-}$	0.69 ± 0.01
	NO_3^-	0.67 ± 0.02
	O_2	0.96 ± 0.03

Anaerobic respiration of tetrathionate as well as nitrate augmented the growth yield on each of the three energy sources (Table 2). This augmentation was highly significant. The yield was 10.8 g/mol growing on pyruvate anaerobically without external electron acceptor. This value is very close to the average value of 10.5 g others have reported (Bauchop & Elsdén, 1960) as growth yield corresponding to 1 mol of ATP. Thus, the number of ATP molecules formed/molecule of the energy source consumed may be calculated by dividing the molar growth yields by 10.8. It follows from such calculations (Table 2) that tetrathionate respiration increased the amount of ATP produced per unit of energy source consumed.

Table 2. *Influence of different exogenous electron acceptors on the growth of Citrobacter*

For details of experimental procedures, see text.

Source of energy and electrons	Exogenous acceptor of electrons	Molar growth yield $Y_{\text{energy source}}$ (g/mol)	$Y_{\text{energy source}}/10.8$ (mol ATP/mol energy source)	Specific metabolic rate (mol substrate consumed/g dry wt/h $\times 10^3$)
Glucose	None	45.0 ± 0.0	4.2	11.3
	$S_4O_6^{2-}$	69.7 ± 2.7	6.5	11.8
	NO_3^-	65.8 ± 1.3	6.1	11.4
	O_2	96.1 ± 3.9	8.9	10.3
Galactose	None	37.2 ± 1.0	3.4	9.4
	$S_4O_6^{2-}$	56.0 ± 1.0	5.2	9.8
	NO_3^-	63.3 ± 2.3	5.9	8.7
	O_2	99.1 ± 1.9	9.2	7.8
Pyruvate	None	10.8 ± 0.6	1.0	38.9
	$S_4O_6^{2-}$	18.3 ± 0.8	1.7	37.7
	NO_3^-	17.6 ± 1.0	1.6	38.1
	O_2	58.3 ± 2.8	5.4	16.5

Table 3. *Molar growth yields and specific rates of reduction of tetrathionate and nitrate in cultures of Citrobacter*Yields expressed in g dry wt/g-ion electron acceptor; metabolic quotients in g-ion reduced/g dry wt bacteria/h $\times 10^3$. For experimental details see text.

Source of energy and electrons	$Y_{\text{tetrathionate}}$	Y_{nitrate}	$q_{\text{tetrathionate}} \times 10^3$	$q_{\text{nitrate}} \times 10^3$
Glucose	88.2	140.7	9.3	5.3
Galactose	43.3	70.3	12.7	7.8
Pyruvate	42.4	64.2	16.3	10.4

Table 4. *Specific growth rate of Citrobacter in minimal medium containing lactate, succinate or glycerol and different exogenous electron acceptors*Cultures as for Table 1 but in medium M_0 , without organic nitrogenous supplements.

Exogenous electron acceptor	Specific growth rate (h^{-1}) on		
	Lactate	Succinate	Glycerol
None	0.00	0.00	0.00
$S_4O_6^{2-}$	0.22	0.05	0.21
NO_3^-	0.08	0.07	0.38
O_2	0.51	0.53	0.51

The last column of Table 2 shows that the specific metabolic rate of glucose and galactose consumption was practically independent of the presence and character of exogenous electron acceptor. This means that the external electron acceptors increased the growth yield and the growth rate practically to the same extent, and that the Pasteur effect, i.e. the relative inhibition of the rate of carbohydrate utilization, occurred. Anaerobically, the specific metabolic rate of pyruvate utilization was more than three times greater than the rate of hexose consumption, but aerobically it was less and approached the rate of hexose dissimilation.

Table 3 shows the growth yields/mol of tetrathionate or nitrate reduced. The specific

metabolic rates of tetrathionate and nitrate reduction were lowest on glucose and highest on pyruvate. The rate of tetrathionate reduction was nearly twice as high as the rate of nitrate reduction. Tetrathionate, like nitrate and oxygen, enabled growth on lactate. Analogous results were found with other non-fermentable carbon and electron sources, succinate and glycerol (Table 4).

DISCUSSION

Three main facts result from our experiments: the reduction of tetrathionate enhances the specific growth rate, raises the growth yield and enables growth on non-fermentable carbon sources. In this respect tetrathionate resembles nitrate and oxygen, and therefore we conclude that our results justify the use of the term 'anaerobic respiration' for the reduction of tetrathionate that is analogous to the reduction of nitrate.

Growth yields/mol of tetrathionate or nitrate reduced were very high (Table 3), possibly because growth by fermentation and growth at the expense of tetrathionate or nitrate respiration took place simultaneously. If no mutual inhibition exists between anaerobic respiration and fermentation there are two possible ways to correct these values. First, only the increase in the specific growth rate should be related to the specific metabolic rate of tetrathionate reduction. Thus,

$$Y'_{\text{tetrathionate}} = \Delta\mu/q_{\text{tetrathionate}}$$

$$[(\Delta g/g/h)/(\text{mol } S_4O_6^{2-}/g/h)] = \Delta g \text{ of bacterial dry wt formed/mol of } S_4O_6^{2-} \text{ reduced}].$$

Secondly, the specific rate of tetrathionate reduction may be related to the specific rate of energy source utilization,

$$q_{\text{tetrathionate}}/q_{\text{energy source}} = Q_{\text{tetrathionate}}$$

$$(\text{mol of tetrathionate reduced/mol of energy source consumed}).$$

The increase in growth yield/mol of energy source consumed should be related to this value, thus

$$Y''_{\text{tetrathionate}} = \Delta Y_{\text{energy source}}/Q_{\text{tetrathionate}}$$

$$[(\Delta g/\text{mol energy source})/(\text{mole } S_4O_6^{2-}/\text{mol energy source})] = \Delta g \text{ of bacterial dry wt formed/mol of } S_4O_6^{2-} \text{ reduced}].$$

Application of both these equations leads to very similar values (Table 5). In the presence of glucose net bacterial dry wt formed/mol of tetrathionate reduced is close to 32 g/mol. Three moles of ATP are generated/mol of tetrathionate reduced, if 10.8 g of bacterial mass formed is taken as equivalent to 1 mol of ATP (Stouthamer, 1962). However, only half this yield was found when galactose or pyruvate replaced glucose. The efficiency of energy production by nitrate respiration was about 50% higher than that by tetrathionate respiration (Table 5).

The formation of ATP during electron transfer to tetrathionate may be predicted from thermodynamics. The standard free energy change $\Delta G'$, pH 7.0, for reoxidation of $NADH_2$ by oxygen, nitrate or tetrathionate, respectively, may be calculated from the difference in standard oxidation-reduction potentials of the two reacting half-cells (Table 6). Standard oxidation-reduction potential of the half cell

$E_0 = +0.17V$ at pH zero (Perelman, 1960). This potential is independent of pH (Slater, 1960); thus $E_0 = E'_0$ (pH 7) = $+0.17V$. Assuming that the dissociation constants of the oxidized and reduced forms do not differ substantially the correction for dissociation may

Table 5. *Corrected growth yields for Citrobacter grown with various substrates*

Data from Table 3 were corrected to allow for fermentation reactions during reduction of electron acceptors. For symbols and equations, see text.

Source of energy and electrons	Y' tetrathionate ($\Delta\text{g/mol S}_4\text{O}_6^{2-}$)	Y' nitrate ($\Delta\text{g/mol NO}_3^-$)	Y'' tetrathionate ($\Delta\text{g/mol S}_4\text{O}_6^{2-}$)	Y'' nitrate ($\Delta\text{g/mol NO}_3^-$)
Glucose	33.3	45.3	31.3	45.2
Galactose	15.7	25.6	14.5	29.0
Pyruvate	16.6	24.0	17.4	25.2

Table 6. *Standard free energy changes $\Delta G'$ (pH 7) of aerobic respiration, respiration of nitrate and respiration of tetrathionate*

Oxido-reduction	E'_0 (pH 7) (V)	$\Delta E'_0$ (V)	$\Delta G'$ (pH 7) (cal/mol)	70 % $\Delta G'$ (cal/mol)	ATP (mol/mol)
$\frac{1}{2}\text{O}_2 + 2\text{H}^+ + 2e \rightleftharpoons \text{H}_2\text{O}$	+0.82	—	—	—	—
$\text{NADH} + \text{H}^+ \rightleftharpoons \text{NAD}^+ + 2\text{H}^+ + 2e$	-0.32	—	—	—	—
$\text{NADH} + \text{H}^+ + \frac{1}{2}\text{O}_2 \rightleftharpoons \text{NAD}^+ + \text{H}_2\text{O}$	—	+1.14	-52 000	-36 400	3.0
$\text{NO}_3^- + 2\text{H}^+ + 2e \rightleftharpoons \text{NO}_2^- + \text{H}_2\text{O}$	+0.42	—	—	—	—
$\text{NADH} + \text{H}^+ \rightleftharpoons \text{NAD}^+ + 2\text{H}^+ + 2e$	-0.32	—	—	—	—
$\text{NADH} + \text{H}^+ + \text{NO}_3^- \rightleftharpoons \text{NAD}^+ + \text{NO}_2^- + \text{H}_2\text{O}$	—	+0.74	-34 000	-23 800	2.0
$\text{S}_4\text{O}_6^{2-} + 2\text{H}^+ + 2e \rightleftharpoons 2\text{S}_2\text{O}_3^{2-} + 2\text{H}^+$	+0.17	—	—	—	—
$\text{NADH} + \text{H}^+ \rightleftharpoons \text{NAD}^+ + 2\text{H}^+ + 2e$	-0.32	—	—	—	—
$\text{NADH} + \text{H}^+ + \text{S}_4\text{O}_6^{2-} \rightleftharpoons \text{NAD}^+ + 2\text{S}_2\text{O}_3^{2-} + 2\text{H}^+$	—	+0.49	-22 500	-15 750	1.3

be neglected and the value $E'_0 = +0.17\text{V}$ may be used. Lehninger (1965) reported that the free energy change $\Delta G'$ resulting from ATP hydrolysis under intracellular conditions at pH 7 is 12 000 cal/mol. When 3 mols of ATP are formed per g-atom of oxygen reduced, the efficiency of energy conservation in aerobic respiration is 70 %. If the same efficiency is also true for both kinds of anaerobic respiration, the quantity of ATP corresponding theoretically to nitrate or tetrathionate reduction may be calculated (Table 6, last column). The formation of ATP as a result of tetrathionate respiration amounts to two-thirds that resulting from nitrate respiration.

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