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Growth of *Wolinella succinogenes* with polysulphide as terminal acceptor of phosphorylative electron transport

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Abstract. Polysulphide was formed according to reaction (1), when tetrathionate was

$$S_4O_6^{2-} + HS^- \rightarrow 2S_2O_3^{2-} + S(O) + H^+ \quad (1)$$

added to an anaerobic buffer (pH 8.5) containing excess sulphide. S(O) denotes the zero oxidation state sulphur in the polysulphide mixture S_n^{2-}. The addition of formate to the polysulphide solution in the presence of *Wolinella succinogenes* caused the reduction of polysulphide according to reaction (2). The bacteria grew in a medium containing formate and sulphide,

$$HCO_2^- + S(O) + H_2O \rightarrow HCO_3^- + HS^- + H^+ \quad (2)$$

when tetrathionate was continuously added. The cell density increased proportional to reaction (3) which represents the sum of reactions (1) and

$$HCO_2^- + S_4O_6^{2-} + H_2O \rightarrow HCO_3^- + 2S_2O_3^{2-} + 2H^+ \quad (3)$$

(2). The cell yield per mol formate was nearly the same as during growth on formate and elemental sulphur, while the velocity of growth was greater. The specific activities of polysulphide reduction by formate measured with bacteria grown with tetrathionate or with elemental sulphur were consistent with the growth parameters. The results suggest that *W. succinogenes* grow at the expense of formate oxidation by polysulphide and that polysulphide is an intermediate during growth on formate and elemental sulphur.

Key words: Sulphur respiration – Polysulphide – Electron transport – *Wolinella succinogenes*

Introduction

Wolinella succinogenes has been shown to grow at the expense of the reduction of elemental sulphur by formate (Macy et al. 1986; Schröder et al. 1988). Since elemental sulphur is nearly insoluble in water (5 µg/l), a soluble intermediate is expected to be formed from sulphur which serves as the direct acceptor in the bacterial formate oxidation. As polysulphide is known to form from elemental sulphur and sulphide, it has been suggested that polysulphide would represent this intermediate (Wloczyk et al. 1989). In this communication the question is investigated whether *W. succinogenes* grown with elemental sulphur, catalyzes the oxidation of formate by polysulphide and whether the bacteria grow at the expense of this reaction.

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Abbreviations: S(O), zero oxidation state sulphur in polysulphides; Tris/HCl, 2-amino-2-hydroxymethyl-1,3-propanediol, pH adjusted by HCl addition; ε, extinction coefficient; A, absorbance

Methods

Growth of W. succinogenes with tetrathionate and sulphide

The growth medium (3 l, 37°C) contained 60 mM sodium formate, 30 mM KH_2PO_4, 24 mM sodium acetate, 6 mM NH_4Cl, 2 mM Na_2S, 0.4 mM $MgCl_2$, 0.4 mM glutamate, 0.2 mM $CaCl_2$ and the trace element solution SL8 (1 ml/l culture) given by Pfennig and Trüper (1981). The medium was adjusted to pH 8.5, flushed with N_2 and inoculated (10%) with a culture grown on formate and sulphur (Schröder et al. 1988). An anaerobic solution of $Na_2S_4O_6$ (1 M) was continuously added using a peristaltic pump.

Activity of polysulphide reduction by formate

The activity was measured using the absorbance difference of polysulphide at 360 and 550 nm with a dual wavelength spectrophotometer. The cuvette (0.5 cm optical path length) contained an anaerobic solution with 0.5 M Tris/HCl, pH 8.5, 10 mM sodium formate and 3 mM Na_2S. After the addition of 1 mM $Na_2S_4O_6$, the reaction was started by the addition of bacteria (up to 0.1 g/l bacterial protein). An absorbance decrease of 0.36 cm^{-1} corresponded to the reduction of 1 mM polysulphide-sulphur S(O) or the oxidation of 1 mM formate.

Determination of thiosulphate and polythionates

Thiosulphate was measured by iodometric titration after sulphide and polysulphide (reaction a) had been removed by the addition of zinc acetate in

$$S_{n+1}^{2-} + Zn^{2+} \rightarrow ZnS + n/8\,S_8 \quad (a)$$

tenfold molar excess, and centrifugation. Thiosulphate and polythionates were also determined by quantitative ion chromatography (Steudel and Holdt 1986; Steudel et al. 1989).

Determination of sulphide

Sulphide including polysulphide was measured as the difference of total I_2 consumption and thiosulphate content. One mol I_2 is consumed per mol sulphide or polysulphide (reaction b).

$$S_n^{2-} + I_2 \rightarrow n/8\,S_8 + 2I^- . \quad (b)$$

Protein

Protein was determined using the biuret method with KCN (Bode et al. 1968).

Determination of formate

Formate was measured photometrically using NAD-linked formate dehydrogenase (Bergmeyer 1974).

Results

The polysulphide solution

Polysulphides are formed by the dissolution of elemental sulphur in aqueous sulphide solutions according to reaction (c) (Gerischer 1949). Formally,

$$n/8\,S_8 + HS^- \rightarrow S_{n+1}^{2-} + H^+ \quad (c)$$

polysulphides may be regarded as being made up of a sulphide dianion and n atoms of sulphur in the zero oxidation state, abbreviated as S(O). As indicated by reaction (c), the amount of elemental sulphur dissolved is a function of the sulphide concentration and the pH. Under conditions compatible with bacterial growth (pH 8.5 and 3.8 mM sulphide) maximally 6.4 mmol/l elemental sulphur can be dissolved. Thus polysulphides are sufficiently stable to function as bacterial substrate.

Solutions with known concentrations of polysulphide-sulphur S(O) are prepared more conveniently by the addition of tetrathionate to anaerobic solutions of sulphide (reaction d). Formally, part of the sulphide is

$$n\,S_4O_6^{2-} + (n+1)\,HS^- \rightarrow 2n\,S_2O_3^{2-} + S_{n+1}^{2-} + (n+1)\,H^+ \quad (d)$$

oxidized by tetrathionate to elemental sulphur which reacts with the excess sulphide to give polysulphides. Upon the addition of 1 mM tetrathionate to solutions (pH 8.5) containing 3–5 mM sulphide, 2 mM of thiosulphate are formed and 1 mM sulphide disappear. Thus the equilibrium of reaction (d) is well on the right side. The absorption spectra of the reaction mixtures with tetrathionate at a given pH (Fig. 1) are identical with those of equimolar polysulphide solutions obtained by the dissolution of elemental sulphur with sulphide or otherwise (Schwarzenbach and Fischer 1960; Giggenbach 1972).

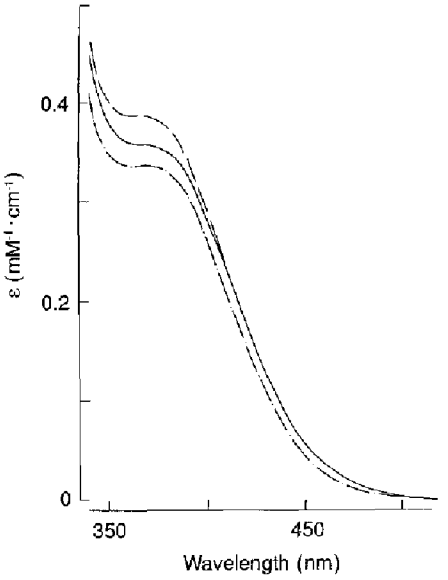

Fig. 1. Spectra of polysulphide solutions at pH 8.0 (– – –), 8.5 (——) or 9.0 (— · —). The sample and the reference cuvette contained anaerobic solutions with 0.5 M Tris/HCl and 5 mM Na_2S. The spectra were taken after the addition of 1 mM $Na_2S_4O_6$ to the sample cuvettes. Extinction coefficients (ε) refer to the amounts of $Na_2S_4O_6$ added

Successive additions of tetrathionate (up to a molar ratio tetrathionate/sulphide of about 0.3) to a sulphide solution (3–5 mM, pH 8.5), caused a linear increase of the absorbance at 360 nm (0.36 cm^{-1} per mM tetrathionate). Thus at a given pH the extinction coefficient per mol polysulphide-sulphur S(O) was independent of the S(O)/sulphide ratio, in agreement with the evaluation given in the Discussion section. However, the extinction coefficient varied with the pH of the solution. At 360 nm it was 16% greater with the pH at 8 than at pH 9 (Fig. 1). According to Giggenbach (1972) this is due to a greater proportion of pentasulphide relative to tetrasulphide at pH 8 (see Discussion). In summary, the content of polysulphide-sulphur S(O) can be adjusted by the amount of tetrathionate added to the sulphide solution. The formation or consumption of polysulphide-sulphur can be recorded photometrically at 360 nm with the pH of the solution kept constant at 8.5.

Reaction of pentathionate with sulphide

Pentathionate was earlier reported to serve as acceptor in the formate oxidation catalyzed by *Wolinella succinogenes* (Wloczyk et al. 1989). Therefore, it was of interest to find out whether pentathionate or higher polythionates may occur in mixtures of sulphide and tetrathionate. For this purpose we have reacted solutions of potassium pentathionate with aqueous sodium sulphide (freshly recrystallized) and monitored the products by ion chromatography. At a molar ratio of exactly 1:1 the only products were thiosulphate and elemental sulphur which immediately precipated from the solution and was filtered off (0.45 µm filter). In a typical experiment

Table 1. Formation of polythionates from aqueous pentathionate (15 mM) and hydrogensulphide (1.5 mM) at 20°C and buffered at pH 8.2. Given are the areas of the chromatographic peaks which are linear proportional to the corresponding concentrations. $K_2S_5O_6$ was synthesized according to Göbel (1988)

Reaction time	$S_2O_3^{2-}$	$S_4O_6^{2-}$	$S_5O_6^{2-}$	$S_6O_6^{2-}$
1 min	1.59	0.21	29.17	0.60
15 min	1.64	0.47	27.82	6.31
45 min	1.71	1.15	25.91	5.95
1 day	2.41	10.73	12.12	4.04
6 days	3.59	11.64	7.40	1.38

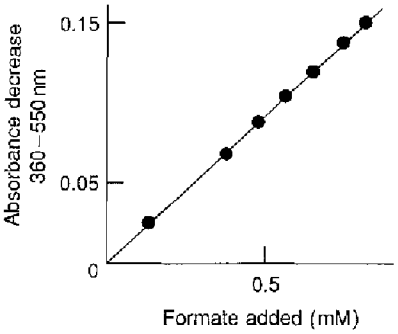

Fig. 2. Titration with formate of the absorbance of a polysulphide solution in the presence of *W. succinogenes*. The anaerobic suspension (37°C and pH 8.5) contained 0.5 M Tris/HCl, 3.2 mM Na_2S, 1 mM $Na_2S_4O_6$ and *W. succinogenes* (94 mg bacterial protein/l) grown with formate and tetrathionate

14.9 mM $S_5O_6^{2-}$ and 14.9 mM HS^- dissolved in a Tris/HCl buffer at pH 8.2 and 20°C yielded 29.1 mM $S_2O_3^{2-}$ within 15 min according to Eq. (e). This

$$S_5O_6^{2-} + HS^- \rightarrow 2\,S_2O_3^{2-} + 2/8\,S_8 + H^+ \qquad \text{(e)}$$

result suggested that pentathionate, like tetrathionate, was not stable with sulphide present in equimolar amounts or in excess. This should also pertain to higher polythionates (see Discussion). Therefore, these compounds should not be present in significant amounts in the polysulphide solution prepared with tetrathionate.

When sulphide was reacted with pentathionate in a tenfold molar excess, only small amounts of elemental sulphur precipitated and various polythionates $S_nO_6^{2-}$ ($n = 4-7$) were formed in addition to thiosulphate (Table 1). The reaction mechanism will be discussed below.

Polysulphide reduction by formate

In the experiment depicted in Fig. 2 *W. succinogenes* were suspended in the polysulphide solution (pH 8.5) prepared with 1 mM tetrathionate, and the absorbance of the suspension was titrated with smaller amounts of formate. Upon each addition of formate, the absorbance decreased according to an apparent first-order reaction which attained equilibrium after a few min (not shown). The

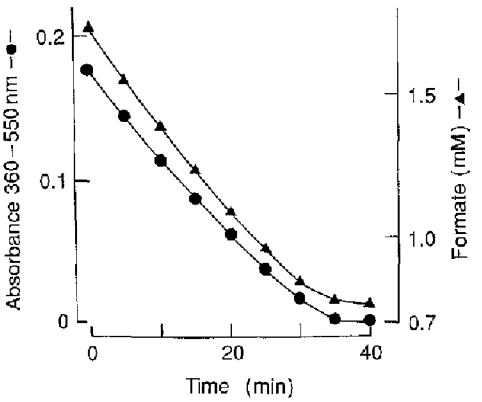

Fig. 3. Polysulphide absorbance and formate concentration as functions of the reaction times in the presence of *W. succinogenes*. The anaerobic reaction mixture (37°C and pH 8.5) contained 0.5 M Tris/HCl, 3.2 mM Na_2S, 1 mM $Na_2S_4O_6$ and 2 mM formate. The reaction was started by the addition of *W. succinogenes* (26 mg bacterial protein/l) grown with formate and tetrathionate

absorbance change was a linear function of the amount of formate added. The slope of the line (0.36 cm^{-1} per mM formate) corresponded to the extinction coefficient of polysulphide-sulphur S(O). Thus the amount of polysulphide-sulphur reduced was equivalent to that of formate added. The difference spectrum of the suspension (before *minus* after formate addition) in the region between 350 and 550 nm (not shown) was identical with the spectrum of polysulphide (Fig. 1). This suggested that the bacteria catalyzed the reduction of polysulphide by formate according to reaction (f).

$$n\,HCO_2^- + S_{n+1}^{2-} + n\,H_2O \rightarrow n\,HCO_3^- + (n+1)\,HS^- + (n-1)\,H^+. \qquad \text{(f)}$$

As a test on the possible occurrence of intermediates, the reduction of polysulphide was recorded and simultaneously samples were taken at various reaction times and analyzed for formate (Fig. 3). The concentration of formate was found to decrease parallel to the absorbance of polysulphide. The plot of absorbance decrease against formate consumption (not shown) gave a straight line with the same slope as that in Fig. 2. This suggested that polysulphide-sulphur was reduced to sulphide at the rate of formate oxidation. Intermediates of the reaction were not noticed.

Growth of W. succinogenes with tetrathionate and sulphide

W. succinogenes did not grow with formate and tetrathionate. However, growth was observed in a medium containing formate and sulphide (2 mM) when a tetrathionate solution was continuously added using a pump (Fig. 4). The rate of tetrathionate addition was increased according to the cell density, and the pH of the medium was kept between 7.9 and 8.5 by the addition of NaOH. After seven hours of growth, the addition of tetrathionate was stopped. As a consequence, growth, formate consumption and the acidification of the medium ceased.

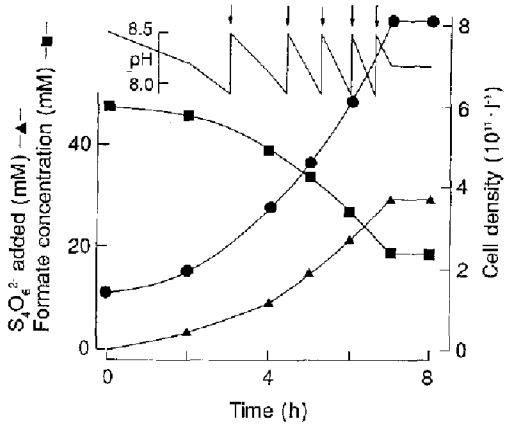

Fig. 4. Growth of *W. succinogenes* with tetrathionate, sulphide and formate. The arrows indicate additions of 10 mM NaOH

Table 2. Growth of *W. succinogenes* with tetrathionate, sulphide and formate. Evaluation of Fig. 4. The ratios formate/tetrathionate, thiosulphate/tetrathionate, and NaOH/tetrathionate were obtained from the straight lines of plots of the respective concentrations measured during growth. The cell yield represents the slope of a plot of the amounts of cellular protein formed during growth against those of formate consumed. Protein was measured using the Lowry method (Lowry et al. 1951) after precipitation of the bacteria from the culture. The protein content of the bacteria was obtained from the plot of protein content against cell density of the culture. The specific growth rate (μ) was calculated from the doubling time (t_d) using $\mu \cdot t_d = \ln 2$

Formate/tetrathionate [mol/mol]	0.96
Thiosulphate/tetrathionate [mol/mol]	1.9
NaOH/tetrathionate [mol/mol]	2
Cell yield (Y) [g protein/mol formate]	1.6
Protein [g/cell]	$6.5 \cdot 10^{-14}$
Doubling time (t_d) [h]	2.3
μ/Y [mmol formate/min per g protein]	3.1

The plot of the amount of tetrathionate added against that of formate consumed (not shown) gave a straight line with a slope of 0.96 mol formate/mol tetrathionate (Table 2). Per mol tetrathionate added, 1.9 mol thiosulphate were formed and 2 mol NaOH had to be added to maintain the pH of the medium between 7.9 and 8.5. The concentration of sulphide (2 mM) did not change during growth. Thus the bacteria grew apparently at the expense of reaction (g).

$$HCO_2^- + S_4O_6^{2-} + H_2O \rightarrow HCO_3^- + 2S_2O_3^{2-} + 2H^+. \quad (g)$$

As the presence of sulphide was required for growth with tetrathionate, it was likely that polysulphide was formed in the culture according to reaction (d) and served as the acceptor of the bacterial formate oxidation according to reaction (f). In agreement with this interpretation, reaction (g) represents the sum of reactions (d) and (f).

During growth, both cell density (Fig. 4) and protein formation (not shown) were linear functions of the amount of formate consumed (Table 2). The cell yield (1.6 g protein/mol formate) was consistent with that measured earlier with *W. succinogenes* growing on formate and elemental sulphur (3.5 g cells/mol formate), since the cellular protein ($6.5 \cdot 10^{-14}$ g/cell, Table 2) amounted to about half the bacterial dry weight ($12 \cdot 10^{-14}$ g/cell, Macy et al. 1986).

Growth started to be exponential 2 h after inoculation with a doubling time of 2.3 h (Fig. 4). From the doubling time and the cell yield, the specific activity of formate oxidation of the growing bacteria was calculated as 3.1 mmol formate/min per g cellular protein.

Table 3. Activities of formate oxidation with various acceptors at 37° C. *W. succinogenes* was grown with elemental sulphur (Schröder et al. 1988) or with fumarate (Bronder et al. 1982) as described. The unit of activity (U) is equivalent to the oxidation of 1 µmol formate/min. Formate dehydrogenase activity was measured photometrically using benzyl viologen as acceptor (Kröger et al. 1979)

Acceptor of formate oxidation	Specific activity of *W. succinogenes* grown with		
	Polysulphide	"Elemental sulphur"	Fumarate
	[U/mg protein]		
Polysulphide	3.9	2.5	0.46
"Elemental sulphur"	—	0.7[a]	0.15[a]
Benzyl viologen	11.6	6.6	1.9

[a] Measured with the bacterial membrane fraction (Schröder et al. 1988)

Activities of polysulphide reduction

The activity of polysulphide reduction with formate was measured photometrically with the bacteria grown with tetrathionate in the presence of sulphide. The activity had its pH optimum at 8.5 and was related to the concentrations of polysulfide or formate according to the Michaelis equation (not shown). The K_m was 25 µM both for polysulphide-sulphur and formate. In the presence of 1 mM S(O) and 10 mM formate, the specific activity (3.9 U/mg cellular protein, Table 3) exceeded that calculated from the growth parameters (see Table 2) by 22%. This result supported the conclusion that polysulphide served as the energy substrate during growth with tetrathionate.

In an earlier publication, the specific activity of formate oxidation with elemental sulphur as acceptor (Table 3) was measured to be much smaller than that calculated from the corresponding growth parameters (1.6 U/mg cellular protein, from 4 h doubling time, Y = 3.5 g cells/mol formate, and 2 g cells corresponding to 1 g cellular protein). This discrepancy could now be explained on the basis that polysulphide served as the acceptor in formate oxidation with elemental sulphur and that the activity observed was limited by the formation of polysulphide from elemental sulphur and sulphide. This explanation was supported by the following result. The activity of polysulphide reduction of the bacteria grown with elemental sulphur was nearly four times greater than that of formate oxidation in the presence of

elemental sulphur (Table 3) and exceeded that calculated from the growth parameters (see above) by 56%.

W. succinogenes grown with fumarate were found to catalyze formate oxidation with elemental sulphur at about one fifth the specific activity of those grown with sulphur (Table 3). Similarly, polysulphide reduction was about five times slower with fumarate-grown bacteria, suggesting that formate oxidation with polysulphide and with elemental sulphur were catalyzed by the same electron transport system. This view was confirmed by comparison of the specific activities of formate dehydrogenase. This activity was three times greater with sulphur- than with fumarate-grown bacteria and was maximum with those grown on polysulphide. In summary, the comparison of the enzymic properties of the bacteria grown under various condition suggests that polysulphide reduction is an essential step in the sulphur respiration of *W. succinogenes*.

Discussion

Mechanism of the polythionate reactions with sulphide

Hydrogen sulphide ions are strong nucleophiles (Davis 1964) which split polythionate anions ($S_4O_6^{2-}$, $S_5O_6^{2-}$) with the formation of thiosulphate, the other product being a sulphanemonosulphonate (Eq. h). The fate of the

$$S_{n+2}O_6^{2-} + HS^- \rightarrow S_2O_3^{2-} + S_nO_3^{2-} + H^+ \qquad \text{(h)}$$

sulphanemonosulphonate is greatly dependent on the reaction conditions. When no excess sulphide over polythionate is present, the $S_nO_3^{2-}$ ions decompose in a series of reaction steps to give thiosulphate and elemental sulphur, the ring size (x) of which varies between 6 and 8 (Eq. i). The

$$S_nO_3^{2-} \rightarrow S_2O_3^{2-} + (n-2) \times S_x \qquad \text{(i)}$$

equilibrium of the overall reaction (h, i) is well on the right side at pH near 8, in agreement with earlier observations (Hansen 1933; Kurtenacker and Goldbach 1927).

When sulphide is present in excess over polythionate, the monosulphonate formed by reaction (h) is split to give polysulphide (k). Polythionates

$$S_nO_3^{2-} + HS^- \rightarrow S_2O_3^{2-} + S_{n-1}^{2-} + H^+ \qquad \text{(k)}$$

or sulphanemonosulphonates are not present at detectable amounts in the reaction mixture, resulting from either pentathionate or tetrathionate.

When sulphide was reacted with pentathionate in excess (see Table 1), the excess pentathionate was attacked by the monosulphonate. This resulted in the formation of polythionates (tetra-, hexa, hepta-, etc.) in a series of reactions, part of which may be summarized by Eq. (l) and (m).

$$S_4O_3^{2-} + S_5O_6^{2-} \rightarrow S_3O_3^{2-} + S_6O_6^{2-} \qquad \text{(l)}$$

$$S_3O_3^{2-} + S_5O_6^{2-} \rightarrow S_4O_3^{2-} + S_4O_6^{2-}. \qquad \text{(m)}$$

Although formate oxidation was observed in the presence of pentathionate and *W. succinogenes* (Wloczyk et al. 1989), pentathionate was probably not the direct acceptor of formate oxidation. Spectrophotometric registration of the reaction in the region between 250 and 350 nm indicated the formation of intermediates absorbing at longer wavelength than pentathionate. These intermediates were probably formed from pentathionate and the sulphide produced by the bacteria during formate oxidation. It could not be decided whether S_n^{2-}, $S_nO_3^{2-}$ or $S_nO_6^{2-}$ were used as the direct acceptors of the bacterial formate oxidation under the experimental conditions applied.

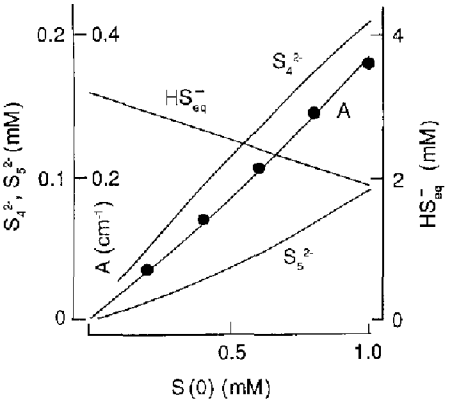

Fig. 5. The concentrations of tetrasulphide and pentasulphide as functions of the content of polysulphide-sulphur S(O). The concentrations were evaluated from the pH, the equilibrium constant of reaction (n) ($4 \cdot 10^{-9}$ M, Giggenbach 1972) and the following two equations: $S(O) = 3\ S_4^{2-} + 4\ S_5^{2-}$ and $HS_{eq}^- = HS_{in}^- - S(O) - S_4^{2-} - S_5^{2-}$. HS_{eq}^- designates the concentration of sulphide at equilibrium and HS_{in}^- that before tetrathionate was added. The absorbance (A) at 360 nm was obtained using the extinction coefficients of tetrasulphide (0.74 mM^{-1} cm^{-1}) and pentasulphide (2.34 mM^{-1} cm^{-1}). The extinction coefficients were calculated from the absorbance of the polysulphide solution at pH 8 and 9 (Fig. 1) and the concentrations evaluated as described. The experimentally determined dots follow a straight line with the slope 0.36 mM^{-1} cm^{-1} (see Results)

The concentrations of tetrasulphide and pentasulphide in the polysulphide solution

According to Schwarzenbach and Fischer (1960) and Giggenbach (1972) tetrasulphide and pentasulphide are the main polysulphide species present in the polysulphide solutions prepared from 3 – 5 mM sulphide (pH 8.5) and up to 1 mM tetrathionate. Longer-chain polysulphides (S_6^{2-}, S_7^{2-}) are expected to be present only in saturated polysulphide solutions in equilibrium with elemental sulphur (Teder 1971; Cocke 1963; Boulegue and Michard 1978).

Tetrasulphide and pentasulphide dismutate according to reaction (n). As

$$3\,S_5^{2-} + HS^- \rightarrow 4\,S_4^{2-} + H^+ \qquad \text{(n)}$$

the equilibrium constant is known (Giggenbach 1972), the concentrations of the two polysulphides can be calculated from that of sulphide, polysulphide-sulphur S(O) and the pH of the solution. In Fig. 5 the concentrations are given as functions of that of polysulphide-sulphur

S(O). The conditions used for the calculation correspond to the titration of a sulphide solution (3.2 mM and pH 8.5) with tetrathionate or to the titration of the resulting polysulphide solution with formate in the presence of bacteria as shown in Fig. 2. Tetrasulphide appears to be the dominant species. The concentrations of both polysulphides are non-linear functions of the concentration of polysulphide-sulphur. This is surprising since the absorbance at 360 nm has experimentally been found to be linearly related to the amount of tetrathionate or formate added. To test this apparent discrepancy, the absorbance resulting from both polysulphides was calculated as a function of the concentration of polysulphide sulphur using the two extinction coefficients (curve "A" in Fig. 5). Comparison shows that the theoretical absorbance follows a slightly non-linear function which is close to the experimental dots with concentrations of S(O) up to 1 mM. At higher concentrations the deviations are beyond experimental error. Such solutions have not been used, therefore, in the experiments described.

The absorbance at 360 nm decreased linearly with formate consumption in the reaction recorded in the experiment of Fig. 3. Considering that the extinction coefficients of the two polysulphides differ greatly (see legend of Fig. 5), this suggests that the dismutation reaction (n) is distinctly faster than the reduction of the polysulphides by formate under the experimental conditions used. A deviation from the linear relationship could possibly occur, if the rate of reduction would be increased by applying the bacteria at 10–100 times greater density. By this type of experiment it is hoped to find out whether the bacteria prefer tetrasulphide to pentasulphide or vice versa.

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