Changes During Long-Term Growth
of *Desulfotomaculum acetoxidans* DSM 771

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Growth rate and concentrations of sulfates, sulfides, proteins and glucosamine were analyzed during long-term (over 60 days) incubation of *Desulfotomaculum acetoxidans* DSM 771. To imitate natural conditions, incubation was done in obligate anaerobic conditions in three series, without stirring or shaking. In the first 2–3 days of incubation (lag phase), only a decrease of the sulfate level occurred. Between days 2 and 7 of incubation (logarithmic increase phase, log phase) the growth rate and levels of proteins and glucosamine increased significantly. Simultaneously the amount of dissimilated hydrogen sulfide began to increase. Hydrogen sulfide content in parallel samples treated with lysozyme was much higher. Between days 7 and 18 a plateau ascribed to the stationary phase was observed. After 2 weeks of incubation a certain reduction of the measured substances was observed, but from days 20 to 24 the growth rate again increased ('post-stationary' phase). The high coefficients of correlation (for individual series 0.6735; 0.7245; 0.8217) between proteins and sulfide levels and control tests done with standards (albumin and Na₂S) suggest that H₂S and probably sulfides react with proteins and presumably with peptidoglycan. This could explain cumulation of sulfide and its decrease in the post-stationary phase.

Key words: *Desulfotomaculum acetoxidans*, long-term growth, sulfide accumulation.

INTRODUCTION

In the 1920s it was already noticed that sulfate-reducing bacteria (SRB) retained their biological activity even when their cultures were stored for several decades (Postgate, 1984). The long persistence of those bacteria in the natural environment may be explained by their ability to utilize many carbon substrates: hydrocarbons, alcohols, simple and fatty acids, amino acids and even aromatic hydrocarbons (Widdel, 1988; Schnel et al., 1989; Aeckersberg et al., 1991; Quatibi et al., 1991; Kuever et al., 1993; Rabus and Widdel, 1995). Several anaerobic bacteria can also use H_2 as an electron donor, while reversible reduction of H^+ to molecular hydrogen is catalyzed by hydrogenases (LeGall and Fauque, 1988). Noguera et al. (1998) worked out a mathematical model showing the theoretical possibility of long maintenance of *Desulfovibrio* species, especially when sulfate and lactate ions in a molar ratio of 0.5 are present in their medium. This ratio is in agreement with the well-known simple equation of sulfate reduction:

$$SO_4^{2-}+2CH_3CHOHCOO^- \rightarrow HS^-+2CH_3COO^-+2CO_2+2H_2O$$

However, *Desulfotomaculum acetoxidans* is capable of complete oxidation of acetate; this can help prolong the maintenance period of those bacteria.

Noguera et al. (1998) showed that consumption of H_2 depends mainly on the value of that molar ratio of sulfate and lactate. Consumption of H_2 ceases completely when the ratio diminishes to 0.38. However, Raskin et al. (1996) found that co-cultures of SRB and methanogenic bacteria could be maintained for over 2 years. Interestingly, addition of sulfate after such a long period did not cause immediate dissimilation of H_2S.

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We investigated changes occurring in the culture medium during long-term incubation in conditions resembling natural ones (without shaking or stirring). *Desulfotomaculum acetoxidans* was chosen for the investigations, as Gram-positive bacteria are rather rare among SRB. Previous observations indicated the suitability of this species for such a study.

MATERIALS AND METHODS

GROWTH OF BACTERIA

Desulfotomaculum acetoxidans strain DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen) 771 was grown for 66 days at room temperature (19–24˚C). The culture medium contained 42.24 mM sodium lactate and 21.12 mM sodium sulfate as substrates. The medium contained the following chemical compounds (per liter): 0.2 g KH_2PO_4 (1.15 mM), 0.3 g KCl (4.02 mM), 0.3 g NH_4Cl (5.61 mM), 0.25 g $CaCl_2·6H_2O$ (1.13 mM $CaCl_2$), 0.4 g $MgCl_2·6H_2O$ (1.97 mM $MgCl_2$), 5.0 g NaCl (85.55 mM) and trace elements according to the Deutsche Sammlung von Mikroorganismen prescription. Before the cold medium was autoclaved its pH was adjusted to $7.0 ± 0.2$. After autoclaving and inoculation the medium was immediately covered with a liquid paraffin layer ~3 mm thick. During incubation, pH and OD_{436} (Cypionka, 1987) were measured. Though previously obtained values recalculated from measured OD_{436} data into real numbers of bacteria were available, it was decided to use the following formula for estimation of the growth rate (GR):

$$GR = \tau_x / \tau_0$$

where:

τ_0 – degree of turbidity at τ_0 (GR on the day of inoculation equals 1.00),

τ_x – degree of turbidity on consecutive days of incubation (τ_x).

CHEMICAL ANALYSIS

Quantitative determinations of SO_4^{2-}, H_2S, proteins and glucosamine were made for three series of experiments.

Sulfate was determined by the nephelometric method with 2-aminoperimidine hydrobromide (Williams, 1985). To eliminate proteins, all samples were prepared by boiling 1 ml samples with a double volume of methanol, evaporating to 0.5 ml, and centrifuging. The supernatants were collected carefully, and precipitates were rinsed with 1.0 ml water and centrifuged again. The sulfate determinations were made after combining both supernatants.

Hydrogen sulfide was measured by the spectrophotometric methylene blue method (Fago and Popowsky, 1949). Two kinds of samples were assayed: one treated with lysozyme (1 mg ml^{-1}) for 24 h at room temperature, and the other without lysozyme treatment.

Proteins were estimated by Lowry's method (Lowry et al., 1951). The assays were carried out for filtered (filter 0.3 μm porosity) and unfiltered samples.

Glucosamine was estimated quantitatively by the modified Elson-Morgan method (Oguchi and Oguchi, 1979) from two parallel samples: filtered (as above) and not filtered. All samples were treated with lysozyme (as above).

All reagents were analytical quality, from Merck or Fluka.

Colorimetric determinations were made with a CECIL 8020 spectrophotometer, and nephelometric measurements with Specol 11 equipment with a TK attachment.

RESULTS

GROWTH RATE

The results on growth of *Desulfotomaculum acetoxidans* DSM 771 presented here are averages from 6 experiments performed in 1999–2000. The high values of the standard deviation for growth rate ($± 107.916 × 10^4.ml^{-1}$; 37.84% – Tab. 1) calculated at the day of inoculation (t_0) could be explained by variation of the growth rate of the investigated bacterial species in different seasons of the year.

Changes in the growth rate calculated according to the given formula are nearly identical with those recalculated for the number of bacteria. The applied method is faster and less cumbersome, and it allows easy comparison with other parameters presented in the graphs.

The low growth rates measured in the first two days of incubation should be ascribed to the lag phase (Fig. 1). During the next days (days 3 to 7) the growth rate index increased from 1.2 to 2.1, indicating the logarithmic or log phase. Later the number

TABLE 1. Statistical analysis of values for choice days (t_n) during long-term incubation

Incubation time	Growth rate	H_2S [mM] + lysozyme	Proteins [g l^{-1}] not filtred	GlcNH$_2$ [g l^{-1}] not filtred
t_0	285.17 [.10^7 l^{-1}] /^{a} ±107.916 (37.84%)	0.002/^{a} ± 0.001 (50.00%)	0.220/^{a} ± 0.147 (66.82%)	0.252/^{a} ± 0.135 (53.57%)
t_2	0.9098/^{a} ± 0.211 (23.19%)	0.687/^{a} ± 0.629 (91.56%)	0.796/^{a} ± 0.507 (63.69%)	0.572/^{a} ± 0.267 (46.75%)
t_3	1.9338/^{a} ± 1.067 (55.20%)	1.500/^{a} ± 0.984 (65.60%)	0.916/^{a} ± 0.594 (64.85%)	0.665/^{a} ± 0.201 (30.23%)
t_4	2.0936/^{a} ± 0.658 (31.43%)	1.543/^{a} ± 0.489 (31.74%)	0.909/^{a} ± 0.459 (50.49%)	0.532/^{a} ± 0.093 (17.48%)
t_7	1.8672/^{a} ± 0.576 (30.85%)	6.484/^{a} ± 2.007 (30.96%)	2.525/^{a} ± 0.909 (36.00%)	0.881/^{a} ± 0.383 (43.52%)
t_8	2.0112/^{a} ± 0.481 (23.92%)	12.821/^{a} ± 4.828 (37.66%)	2.184/^{a} ± 0.586 (26.83%)	0.695/^{a} ± 0.214 (30.79%)
t_{10}	1.8744/^{a} ± 0.433 (23.11%)	14.257/^{a} ± 6.778 (47.54%)	1.924/^{a} ± 0.442 (22.97%)	0.470/^{a} ± 0.038 (8.09%)
t_{12}	1.8293/^{a} ± 0.447 (24.44%)	4.341/^{a} ± 2.249 (60.27%)	2.019/^{a} ± 0.361 (17.88%)	0.255/^{a} ± 0.123 (48.24%)
t_{14}	1.9881/^{a} ± 0.678 (34.10%)	8.105/^{a} ± 4.200 (51.82%)	1.552/^{a} ± 0.048 (3.09%)	0.238/^{a} ± 0.113 (47.48%)
t_{17}	1.4553/^{a} ± 0.579 (39.79%)	2.337/^{a} ± 0.920 (39.37%)	1.138/^{a} ± 0.111 (9.75%)	0.275/^{a} ± 0.037 (13.45%)
t_{21}	1.5144/^{a} ± 0.626 (41.34%)	0.249/^{a} ± 0.126 (50.40%)	0.880/^{a} ± 0.154 (17.50%)	0.280/^{a} ± 0.037 (13.21%)
t_{24}	1.8313/^{a} ± 1.039 (56.74%)	0.468/^{a} ± 0.271 (57.91%)	0.616/^{a} ± 0.128 (20.74%)	0.245/^{a} ± 0.058 (23.67%)
t_{27}	1.2451/^{a} ± 0.573 (46.03%)	0.781/^{a} ± 0.265 (33.94%)	0.241/^{a} ± 0.058 (24.06%)	0.272/^{a} ± 0.080 (29.41%)
t_{35}	1.3262/^{a} ± 0.893 (67.38%)	0.062/^{a} ± 0.001 (7.63%)	0.385/^{a} ± 0.016 (4.16%)	0.258/^{a} ± 0.023 (8.91%)
t_{44}	1.1082/^{a} ± 0.468 (42.24%)	0.0403/^{a} ± 0.005 (12.41%)	0.419/^{a} ± 0.025 (5.97%)	0.158/^{a} ± 0.017 (10.76%)
t_{66}	0.7152/^{a} ± 0.135 (18.88%)	0.0122/^{a} ± 0.003 (24.59%)	0.327/^{a} ± 0.039 (11.93%)	0.191/^{a} ± 0.017 (8.90%)

/^{a} - arithmetic mean; ± – standard deviation; (..) – variance.

of bacteria cells decreased, and increased again after some time (stationary phase) (Fig. 1). These results reflect slowed but cyclic growth in the examined strain.

The calculated standard deviation values as well as the growth rate index indicate significant variability of the growth rate, especially in the log and post-stationary phases. (Tab. 1). That is not surprising, considering that the interval between taking cultures from the DSM bank and performing the experiments varied.

The sporulation process in this bacterial strain is also highly delayed, as only negligible numbers of resting spores were found in culture after 80 days of incubation (data not presented).

PROTEINS AND GLUCOSEAMINE

Parallel assays with filtered and unfiltered samples were performed in order to find out whether *Desulfotomaculum acetoxidans* liberates either proteins or glucosamine needed for binding nutrient elements such as iron.

Notwithstanding the differences in amounts of peptides in individual series (Tab. 1), it was noted that up to day 7 of incubation (in the lag and log phases) the increase in the growth rate was accompanied by an increase in the protein level. The content of liberated substances, mainly peptides (and amines) in the supernatants, reached 30%

(Fig. 1). The coefficient of correlation between the growth rate and peptide content for 66-day incubation amounted to 0.4696, and for the stationary and post-stationary phases was 0.4396 (Tab. 2). The reduction of the correlation coefficient seems attributable to the variable rhythm of the two parameters, especially between the days 7 and 14. From day 21 the amounts of protein in filtered and unfiltered samples were similar, and simultaneously diminishing (Fig. 1).
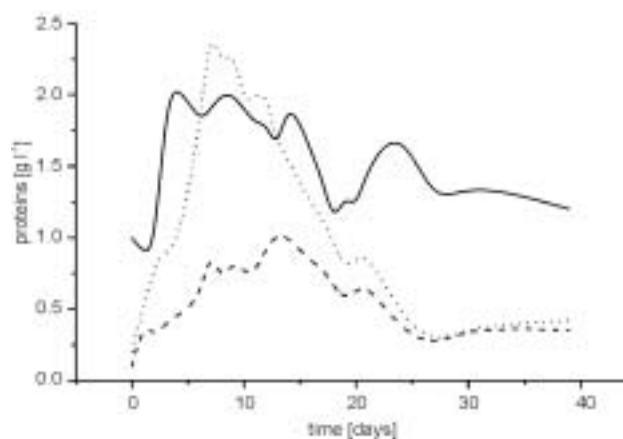

Fig. 1. Changes in growth rate (solid line), filtered protein level (dashed line) and unfiltered protein level (dotted line) during incubation of *Desulfotomaculum acetoxidans*.

TABLE 2. Correlations/[a] between values during long-term incubation

Growth rate and H₂S/b +lysoz.	Growth rate and proteins	Growth rate and glucosamine	H₂S/[b] + lysoz. and proteins	H₂S/[b] + lysoz. and glucosamine	Proteins and glucosamine
0.4638/[c]	0.4690/c	0.1964/c	0.8433/c,e	0.4248/c	04929/[c]
0.5345/[d]	0.4396/[d]	0.4225/[d]	0.8507/[d]	0.6323/[d]	0.7262/[d]

/[a] – on the basis of arithmetic means; /[b] – H₂S (and/or sulfides); /[c] – from 1 to 44 days of incubation; /[d] – from 7 to 27 days of incubation; /[e] – values for individual series were 0.6735; 0.7245; 0.8217.

The results on glucosamine content in samples, especially in unfiltered ones, were quite surprising. The amount of glucosamine, the main component of peptidoglycan, was expected to increase with the increase in numbers of bacteria (not presented). However, the correlation coefficient for the two parameters during the entire period of incubation was only 0.1964 (Tab. 2); during the stationary and post-stationary phases it increased to 0.4225.

SULFATE

Specific methods of sulfate determination require a large volume of samples. The method applied in this work, however precise, unfortunately gives positive reactions with both sulfate derivatives and thiosulfates.

The results from sulfate determination indicate that the amount of this catabolic substrate in the culture medium dropped rapidly. After 3 days of incubation its concentration was 7.54 mM (Fig. 3). Subsequently the reduction of the SO_4^{2-} level was much slower, but after 10 days only traces of sulfate could be found. In the investigated species, sulfate binding was most intensive in the lag phase.

HYDROGEN SULFIDE

Dissimilation of hydrogen sulfide as a final product in the path of the sulfate reduction process was initiated after 2 to 4 days of incubation in the samples treated with lysozyme (Fig. 2). The highest concentration of H₂S in the sample was found between days 5 and ~18 (stationary phase); later it decreased gradually. The concentration of H₂S in samples not treated with lysozyme was measured in μM in the lag and post-stationary phases, and was up to several mM in the stationary phase (Fig. 2). In parallel samples the amount of H₂S was much higher in those treated with lysozyme. Most surprising was that the concentration of dissimilated hydrogen sulfide in samples treated with lysozyme (in

two series conducted 9 months and a year after taking the culture from the DSM bank) and harvested after 7 days of incubation exceeded the amount of sulfate introduced into the nutrient medium. This influenced the arithmetic mean, which was 14.26 mM, but was 21.04 mM in the third series (Tab. 1). Under starvation conditions without sulfate, liberation of H₂S began earlier (after ~30 h) and after 5 days its level reached the highest value of 4.73 mM (Fig. 3). To find the reason for those very high concentrations of H₂S and/or sulfides, the trace amounts of sulfates which could have been introduced together with other components of the nutrient were calculated precisely. However, only 16 μM of the sulfates could be properly confirmed.

Thus the results suggest binding of H₂S and/or sulfides to the cell walls of *Desulfotomavulum acetoxidans*. The investigated species are Gram-positive bacteria, having very large, thick cell walls. Since lysozyme caused liberation of H₂S, it must have been previously absorbed as deposits. Further experiments showed that the same effect of increased H₂S could be achieved by leaving a sample for 24 h with reagents).

The correlation coefficient for H₂S and glucosamine levels calculated for the entire incubation period was 0.4248, but for the stationary and post-stationary phases (when cumulation can occur) it increased to 0.6323 (Tab. 2). The highest coefficients of correlation between H₂S level and protein level were 0.8433 for the whole incubation period and 0.8507 for the stationary and post-stationary phases. Those very surprising values seem to suggest that the peptide bonds participate in the process of H₂S sorption. An additional test was carried out for H₂S cumulation. When the standard albumin solution (5 mg ml⁻¹) was treated with an equal volume of 20 mM Na₂S, the precipitate appeared after only 3 h, giving a positive reaction by the methylene blue method. Colorimetric estimation showed that after the next 24 h the characteristic absorbance (at λ = 665 nm) was higher
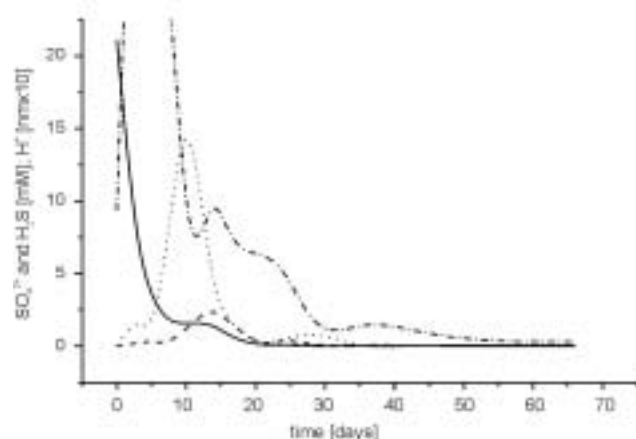
Fig. 2. Changes in concentration of sulfate (solid line), sulfide (samples with lysozyme – dotted line), sulfide (samples without lysozyme – dashed line) and hydrogen protons (dot-dashed line) during incubation of *Desulfotomaculum acetoxidans.*
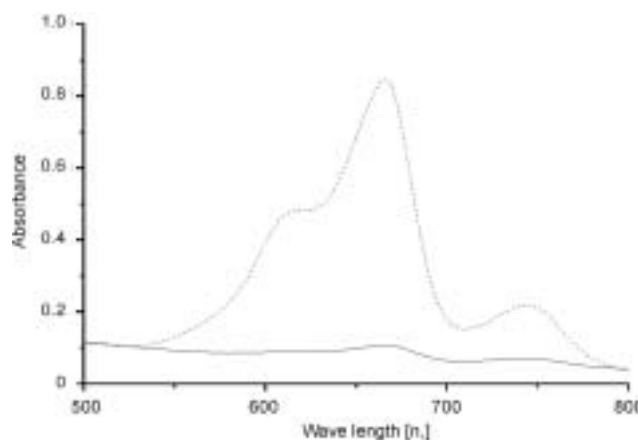

Fig. 3. Absorption spectra of Na_2SO_4-free culture samples after H_2S determination at 30 h (solid line) and 5 days (dashed line) of incubation.
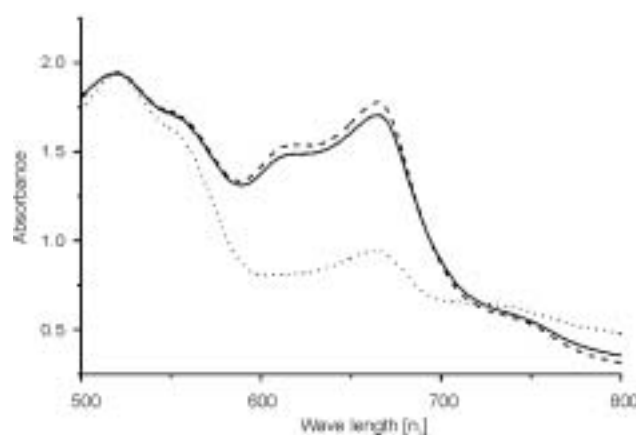

Fig. 4. Absorption spectra of standard albumin (5 mg$\cdot$ml^{-1}) after treatment with equal volumes of 20 mM Na_2S and then methylene blue method reagents: 10 mM Na_2S solution (dashed line); precipitate (dotted line) and precipitate after the next 24 h (solid line).

than before (Fig. 4). Those results indicated that the liberation of sulfides from proteins required at least 24 h of the action of oxygen and acid hydrolysis (final concentration of HCl in the sample was 0.35 mM). This means that proteins and probably peptidoglycan are essential in sulfide cumulation.

Statistical analysis showed immense variation in the liberation and cumulation of H_2S and/or sulfides (Tab. 1). The ability to cumulate H_2S might be dependent on the age of the inoculum.

DISCUSSION

Our investigations suggest that during the lag phase, lasting 2–3 days, mainly processes of sulfate assimilation occur in *Desulfotomaculum acetoxidans* DSM 771 (compare growth rate in Fig. 1 with sulfate concentration in Fig. 2). This seems quite obvious since sulfate is the main metabolic substrate for nearly all sulfate-reducing bacteria. These results confirm other data (e.g., Postgate, 1984; Widdel, 1988; LeGall and Fauque, 1988).

Raskin et. al (1996) also found H_2S dissimilation by SRB delayed versus the time of sulfate addition, even though the bacteria were maintained for two years and no sulfates were added to the medium in the meantime. Moreover, medium free of SO_4^{2-} showed liberation of H_2S, which began earlier (after ~30 h), and reached the highest value of 4.73 mM after 5 days (Fig. 3).

The levels of hydrogen sulfide, much higher in the lysozyme-treated than in the untreated samples (Fig. 2), suggest that only part of this product is liberated into the environment. It should be emphasized that a similar effect of increased H_2S can be obtained by leaving a sample for 24 h. This would mean that hydrogen sulfide is cumulated in the outer cell structures of *Desulfotomaculum acetoxidans,* which is quite probable for Gram-positive bacteria. Cumulation of H_2S might also explain the astonishing high concentration of it (21.04 mM) in the samples treated with lysozyme in the third series after 10 days of incubation (Tab. 1).

In our experiments the whole amount of sulfate was used in the dissimilatory reduction path of SO_4^{2-}; Badziong et al. (1978) found dissimilation of SO_4^{2-} to be ~93%, and Kuever et al. (1993) found up to 80%.

The coefficient of correlation between H_2S and protein levels calculated for the entire incubation period was highest (even over 0.8; Tab. 2). That result seems to suggest that the peptide bonds participate in the process of H_2S sorption. This bonding could explain the decrease of H_2S in the stationary and post-stationary phases (compare Figs. 1 and 2). Additional testing with albumin and Na_2S standards showed bonding of sulfide by proteins. Estimation after the next 24 h showed higher absorbance at λ = 665 nm than before (Fig. 4), indicating that liberation of sulfides from their linkage to proteins requires at least 24 h of the action of oxygen and acid hydrolysis. Further chemical analysis could explain the problem of bonding of sulfides by peptides undergoing the thionation reactions of Lawesson's reagent, which is a source of H_2S (Cava and Levinson, 1985). The more likely reaction, however, is protein S-thiolation, which is reversible (Włodek and Iciek, 2003).

The drop in the level of dissimilated H_2S started from day 10 of incubation (Fig. 2). During that time the growth rate and the amount of proteins decreased (Fig. 1). Since the cultures were not shaken or stirred, the observed decrease might be the result of decantation of bacterial cells. Between days 20 and 24 the growth rate increased again (Fig. 1).

Some authors (Dilling and Cypionka, 1990; Fuseler et al., 1996; Johnson et al., 1997) explained the possibility of growth of SRB in oxidative conditions by their ability to oxidize sulfides and other intermediate metabolites to sulfates by reversing the path of sulfate reduction. Thus, the processes of sulfide oxidation would occur inside the cell, which requires the transport of substances into the cytosol. If it is assumed that H_2S, and/or a sulfide of, for example, sodium, is liberated into an environment outside the cell wall, its concentration would steadily decrease not only because of diffusion but also as a result of transformation into hardly soluble polysulfides. Thus the uptake of sulfides would be difficult and therefore limited. Their cumulation would allow those limitations to be avoided, which in the natural environment is especially important.

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