Sulfate Transport in Normal and S-Deprived *Chlorella fusca*

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Sulfate uptake into the unicellular green alga *Chlorella fusca* Shihira et Krauss strain 211-8b was described as a pH-, temperature- and energy-dependent process. Transport followed triphasic kinetics with K_m-values of 1.3×10^{-5}, 3.9×10^{-5} and 2.8×10^{-4} M. The Hill plot showed coefficients of 1.0 for each uptake isotherm. Sulfate uptake was substantially inhibited by chromate, selenate, sulfite, thiosulfate and tetrathionate. Cysteine and other mercaptides diminished sulfate transport, whereas methionine was not inhibitory. Uptake was sensitive towards inhibitors of photosynthesis and respiration and towards SH-group reagents. Sulfate uptake could be strongly enhanced by polyvalent cations, apparently *via* reduction of the surface potential. Sulfate uptake in S-deprived algae was markedly different from constitutive sulfate uptake. Time kinetics was not linear but showed an initial rapid phase. Uptake was neither multiphasic nor conformed to simple Michaelis-Menten kinetics. Sulfate uptake was hardly affected by pH and temperature in the suboptimal range. The Arrhenius plot was biphasic with a discontinuity at 38 °C.

Introduction

Sulfur is an essential macroelement for algal nutrition, mainly available as sulfate in nature. Sulfate can be utilized as the only sulfur source, showing a rapid transformation to sulfur amino acids, sulfolipids and other S-containing substances. The first step in sulfate utilization is the uptake of the divalent anion into the cell. Sulfate uptake has been characterized in a variety of autotrophic organisms as an energy-dependent and highly specific process. In fungi and higher plant systems, sulfate transport was energized by a $3\,H^+$/sulfate cotransport mechanism, regulated by the intracellular sulfate pool [1, 2].

So far, only limited information is available for sulfate transport systems in green algae. Studies have been reported for the green alga *Chlorella* [3−6]. Due to the small quantities of sulfate assimilated by algae and plants, investigators often used "sulfate starvation conditions" to enhance sulfate transport and incorporation [5, 7−9]. Also the green alga *Chlorella fusca* showed a marked increase of transport rates under sulfate limitation [10]. Sulfate transport rose tenfold after a lag period of 2.5 h, strictly correlated with a depletion of the sulfate pool and the appearance of a sulfonate permease. Addition of external sulfate caused reversion of increased uptake and revealed a half-life of less than 1 h for the altered transport modus [11]. Both enhanced sulfate uptake and the appearance of a new sulfonate uptake system in *Chlorella fusca* were interpreted as specific responses to sulfate limitation [10].

Little is known, however, about the nature of this system for enhanced uptake and the differences between sulfate transport in normal and S-deprived organisms. The following study was initiated to characterize sulfate transport in *Chlorella fusca* under optimal and starvation-induced conditions. We will compare both uptake types and interpret them as a function of the internal sulfate pool.

Materials and Methods

Organism

Chlorella fusca Shihira et Krauss, strain 211-8b was obtained from the algal collection of Göttingen (F.R.G.) and cultured in the SL-1 ("sulfur-less") medium, pH 6.2: 5 mM KNO_3, 2 mM $Mg(NO_3)_2 \times 6$ H_2O, 0.5 mM KH_2PO_4, 0.5 mM K_2HPO_4, 0.25 mM $Ca(NO_3)_2 \times 4$ H_2O, 0.09 mM $EDTA/FeCl_2$ (each),

46 µM H_3BO_3, 12.5 µM $Mn(NO_3)_2 \times 4\ H_2O$, 0.73 µM $ZnCl_2$, 0.31 µM $Cu(NO_3)_2 \times 3\ H_2O$, 0.089 µM $Na_2MoO_4 \times 2\ H_2O$. For exponential growth the medium contained 0.3 mM sodium sulfate. For sulfate starvation conditions Na_2SO_4 was omitted and in some experiments replaced by ethanesulfonate (0.3 mM), a sulfur compound that allows growth under conditions of sulfate starvation [10]. The algae were grown in a Kniese apparatus at 27 °C, continuous light, and a fluence rate of 90 µmol $m^{-2}\ s^{-1}$ (type Osram L-Interna 40 W-39c). 750 ml cultures were run without synchronization in 1 l flasks ($\varnothing = 65$ mm) and gassed with normal air. The cultures were started with a 0.1 ml inoculum of *Chlorella* stock cultures (6×10^6 cells) to minimize the carry over of sulfur compounds. Algal cells were harvested in the exponential growth phase usually 4–5 days after inoculation, S-starved algae were grown for 5 days.

Uptake assays

Cultures were harvested by centrifugation (5 min at $5000 \times g$), washed in sulfur-free medium and resuspended to a density of $1-2 \times 10^8$ cells ml^{-1} in 2 ml of SL-1 medium, pH 6.2. The experiments were started by addition of [^{35}S]sulfate to a final concentration of 0.3 mM and a specific radioactivity of 65 kBq $nmol^{-1}$. Algae were incubated for 5 to 90 min at 27 °C in a Warburg vessel using a "Warburg" apparatus illuminated with Riva D 1 lamps and a fluence rate of 240 µmol $m^{-2}\ s^{-1}$ (400 to 800 nm range). Samples were washed several times by centrifugation and resuspension in S-free medium. The radioactivity accumulated was determined in a liquid scintillation counter and corrected for unspecific adsorption, determined from previously heat-killed cells. Chemicals and equipment ^{35}S-labeled carrier-free sulfate was obtained from Amersham-Buchler (Braunschweig, W. Germany). All other chemicals were purchased as described earlier [12]. The Kniese apparatus for algal cultivation was purchased from Kniese (Marburg, W. Germany).

Results

A) Characterization of the constitutive sulfate uptake in Chlorella fusca

Linearity of sulfate uptake: time course and cell density

Sulfate uptake at 0.3 mM was linear with respect to time for up to 90 min of incubation at 28 °C. Sulfate

uptake was directly proportional to cell density up to 3×10^8 cells ml^{-1} in the assay. Cell densities of $1-2 \times 10^8$ cells ml^{-1} were used for all subsequent uptake studies.

Effect of pH and temperature on sulfate uptake

Both pH and temperature had a pronounced effect on constitutive sulfate uptake in *Chlorella fusca* (Fig. 1, 2). A pH of 9.0 was optimal using 0.01 M Tris/HCl buffer, pH 7.0–10.5 during incubation. In 0.01 M KH_2PO_4/K_2HPO_4 buffer, pH 5.0–8.5 sulfate transport was maximal at pH 8.5 and declined rapidly at lower pH-values confirming an unusually high pH-optimum.

The temperature optimum of sulfate uptake was about 43 °C (Fig. 2). At higher temperatures sulfate uptake decreased strongly, strongly, and transport was completely inactivated after short preincubation above 55 °C. The Q_{10} decreased from 2.0 between 18 °C and 33 °C to 1.7 between 33 °C and 43 °C. From the Arrhenius plot an approximate activation energy of 49.1 kJ mol^{-1} between 18 °C and 43 °C was calculated. The Arrhenius plot was monophasic over the whole temperature range tested, indicating that
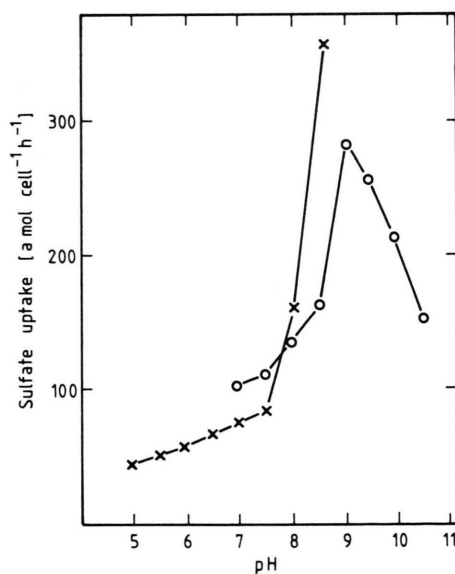

Fig. 1. pH-dependence of constitutive sulfate uptake. Sulfate-grown algae were washed and incubated in 0.01 M KH_2PO_4/K_2HPO_4 buffer (pH 5.0–8.5) or in 0.01 M Tris-HCl buffer (pH 7.0–10.5). Uptake period 30 min. Average of three replicates; average SE 9.0%. ×——× = phosphate buffer; O——O = Tris-HCl buffer.
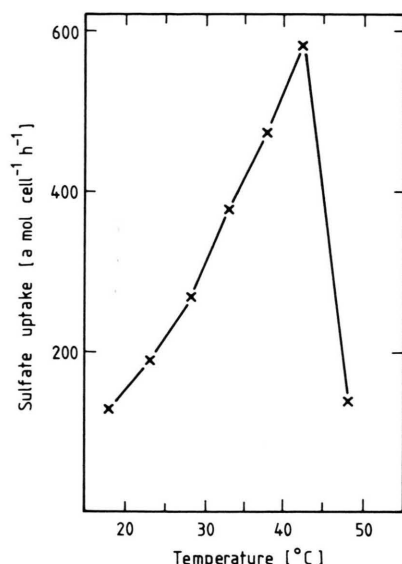
Fig. 2. Temperature dependence of constitutive sulfate uptake. Washed sulfate-grown algae were suspended in the prewarmed S-free medium and assayed for sulfate uptake after 15 min incubation. Average of three replicates; average SE 12.1%.

membranous transition had occurred at lower temperature (13 °C [13]).

Concentration dependence of sulfate uptake

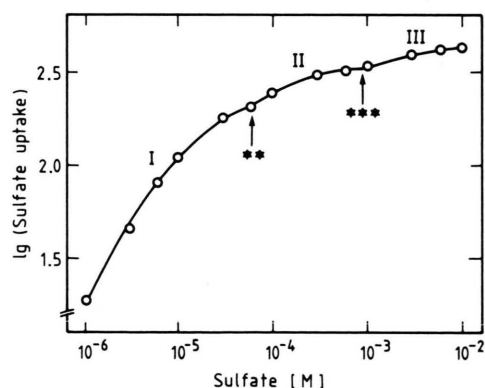The rate of sulfate uptake was determined in the concentration range from 10^{-6} M to 10^{-2} M (Fig. 3).

Fig. 3. Concentration dependence of constitutive sulfate uptake. Uptake period 30 min. Data are expressed as amol cell^{-1} 30 min^{-1}. Average of three replicates; average SE 8%. The roman numerals represent isotherm sections designated in Table I; **, *** refer to transition points.
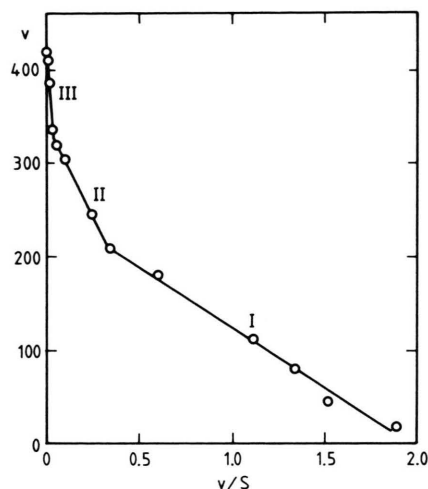
Fig. 4. Eadie-Hofstee plot of sulfate uptake using the data of Fig. 3. Sulfate concentration is given in μM.

The Eadie-Hofstee plot (Fig. 4) showed a triphasic response with K_m-values in increasing order:

Phase I $(1 \times 10^{-6}$ M to 6×10^{-5} M): $K_m = 1.3 \times 10^{-5}$ M
Phase II $(6 \times 10^{-5}$ M to 9×10^{-4} M): $K_m = 3.9 \times 10^{-5}$ M
Phase III $(9 \times 10^{-4}$ M to 1×10^{-2} M): $K_m = 2.8 \times 10^{-4}$ M

Thus, sulfate uptake in the alga *Chlorella fusca* appeared to be multiphasic as in higher plants [14, 15]. Sulfate transport can be described by a single, multiphasic isotherm having three phases. Each phase covered a limited concentration range, obeyed Michaelis-Menten kinetics and was separated from adjacent phases by sharp transition points (see Table I). The uptake data of Fig. 4 and 5 were analyzed

Fig. 5. Hill plot of sulfate uptake as a function of sulfate concentration using the data of Fig. 3. The roman numerals refer to isotherm sections designated in Table I.

Table I. Kinetic constants and transition points for sulfate uptake in *Chlorella fusca*. Calculation according to data presented in Fig. 3 and 4; **, *** refers to transition points.

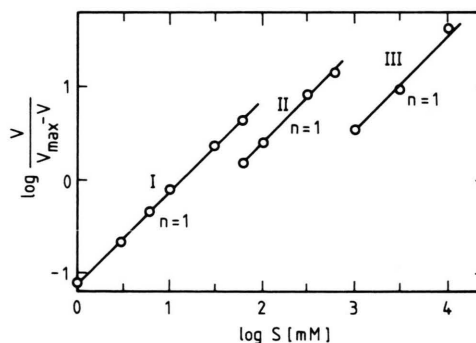| Phase	V_{max} [amol cell^{-1} 30 min^{-1}]	K_m [M]	Transition [M]
I	252 ± 9	$(13 \pm 1) \times 10^{-4}$	
II	339 ± 4	$(39 \pm 2) \times 10^{-4}$	6.0×10^{-5} (chosen)**
III	426 ± 6	$(28 \pm 3) \times 10^{-3}$	9.2×10^{-4} (calc.)***

for cooperativity using the following Hill equation (Fig. 5):

$$\log (V/V_{max} - V) = n \log S - K_m. \tag{1}$$

Each phase has a single slope and Hill coefficients of 1.0.

Substrate specificity of sulfate uptake

The specificity of the sulfate uptake system was tested by competition experiments using various group VI anions of the type XO_4^{2-} (Table II). Greatest inhibition was observed by chromate, where 3 mM resulted in 90% inhibition and 0.03 mM in 55% inhibition. Selenate, which is a distinctly larger anion than sulfate, diminished transport processes with increasing concentrations and caused 74% inhibition at 3 mM. Little inhibition of sulfate transport was observed in the presence of molybdate or tungstate. 3 mM sulfate itself decreased uptake to about 15% according to isotopic dilution (Table II).

Obviously the alga *Chlorella fusca* could not discriminate among anions of type XO_4^{2-} except by the different molecule size itself. Thus, only chromate and selenate compete for sulfate uptake, whereas the significantly larger anions, molybdate and tungstate, do not affect sulfate transport.

Sulfate uptake was further inhibited by thiosulfate and by tetrathionate and its analogue sulfite, using a 10-fold higher concentration as compared to sulfate (Table II). The existence of specific recognition sites for sulfate attachment was further demonstrated by the failure of the monovalent anions chloride, nitrate, methanesulfonate and ethanesulfonate to affect sulfate transport negatively. Also phosphate did not influence sulfate influx in *Chlorella fusca*.

Finally the effects of cysteine, methionine and other SH-containing compounds were studied. Both amino acids are known as end products of sulfur

Table II. Effect of structural analogues and other compounds on sulfate uptake. Washed algae were preincubated for 5 min in the presence of the unlabeled test substance (3 mM) before adding [^{35}S]sulfate to the incubation medium. Uptake period 30 min. All compounds were used as sodium salts, except the amino acids and mercaptenes. Control: 620 amol cell^{-1} h^{-1}. Averages $\pm$ SD ($n = 3$).

Compound [3 mM]		Sulfate uptake [% of control]
None		100
Sulfate		15 ± 2
Sulfite		34 ± 3
Thiosulfate		25 ± 2
Tetrathionate		31 ± 3
Nitrate		98 ± 4
Chloride		115 ± 5
Ethanesulfonate		100 ± 4
Methanesulfonate		103 ± 4
Phosphate		104 ± 5
Cysteine		65 ± 3
Methionine		108 ± 4
Mercaptoethanol		80 ± 5
Cysteamine		65 ± 4
Dithioerythritol		37 ± 4
Chromate	0.03 mM	45 ± 5
	0.3 mM	22 ± 5
	3.0 mM	10 ± 2
Selenate	0.03 mM	90 ± 8
	0.3 mM	53 ± 6
	3.0 mM	26 ± 4
Molybdate	0.03 mM	102 ± 6
	0.3 mM	101 ± 6
	3.0 mM	85 ± 4
Tungstate	0.03 mM	102 ± 10
	0.3 mM	100 ± 5
	3.0 mM	97 ± 5

metabolism. Whereas methionine did not inibit sulfate transport, cysteine diminished transport rates to 65% of the control value, comparable to the influence of other SH-compounds on this uptake system.

Table III. Effect of various inhibitors on sulfate uptake. Washed algae were preincubated for 5 min in the incubation medium containing each compound in the concentration indicated. After adding [^{35}S]sulfate, the Warburg vessels were incubated for 30 min. Substances marked with * were solubilized in ethanol and referred to the ethanol control. Control: 602 amol cell^{-1} h^{-1}. Averages ± SD (n = 3).

Inhibitor	Concn. [mM]	Sulfate uptake [% of control]
None		100
Sodium fluoride	1.0	125 ± 9
Sodium iodoacetate	1.0	150 ± 11
Sodium cyanide	1.0	62 ± 6
Sodium azide	1.0	67 ± 6
DCMU*	1.0	45 ± 6
"Darkness"		51 ± 5
Dibromothymochinon*	0.01	124 ± 7
	0.1	84 ± 6
	1.0	0
2,4-DNP*	1.0	39 ± 4
CCCP*	0.01	20 ± 3
	0.1	0
	1.0	0
DCCD*	0.01	72 ± 7
	0.1	27 ± 3
	1.0	13 ± 4
NEM*	1.0	36 ± 3
PCMBS	1.0	125 ± 9
Ethanol (1%)	117	105 ± 7

Effect of various inhibitors on sulfate uptake

Several metabolic inhibitors were tested for their effects on sulfate uptake (Table III). Sulfate uptake was not diminished by inhibitors of glycolysis (fluoride, iodoacetate), both compounds even increased sulfate transport. Inhibitors of photosynthesis and respiration (cyanide, azide, DCMU and DMBIB) decreased uptake markedly. "Darkness" reduced sulfate uptake to 50% of the light control. The uncouplers 2,4-DNP and CCCP showed substantial inhibition; also the ATPase inhibitor DCCD decreased transport rates. The sulfhydryl group reagent N-ethylmaleimide reduced transport significantly, whereas the non-penetrating thiol reagent PCMBS failed to affect sulfate uptake, excluding the interaction of outer-membrane sulfhydryl groups in the transport process. Ethanol, used to solubilize certain inhibitors, had no effect.

Effect of various cations on sulfate uptake

Most of the metal ions tested had pronounced effects on sulfate uptake (Table IV); especially di- and trivalent cations stimulated uptake 2−3-fold. Although *Chlorella fusca* showed uptake rates already in 1 mM phosphate buffer, divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}) and trivalent ions (Fe^{3+}, Al^{3+}) enhanced transport of sulfate markedly. Nearly identical effects were observed with all divalent and trivalent cations tested, whereas their anions were interchangeable. Monovalent cations (Li^+, Na^+, K^+) had no positive effect on sulfate uptake; Hg^{2+} was inhibitory, probably due to interference with SH-groups.

B) Characterization of the enhanced sulfate uptake under sulfate limitation

Time course

Whereas constitutive sulfate uptake was linear in time up to 90 min incubation, sulfate transport in S-starved cultures followed an unusual pattern (Fig. 6). The initial rate of uptake was very rapid and reached a maximum in less than 7.5 min. This initial rapid uptake was followed by a slow but linear uptake over the next 1 h ("steady-state rate").

Table IV. Effect of various cations on sulfate uptake. Washed algae were preincubated for 5 min in the 3 mM concentrated compound before adding [^{35}S]sulfate to 1 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.5). Uptake period 30 min. Cations were used as the chloride salts; data are not corrected for possible phosphate complexes. Control: 590 amol cell^{-1} h^{-1}. Averages ± SD (n = 3).

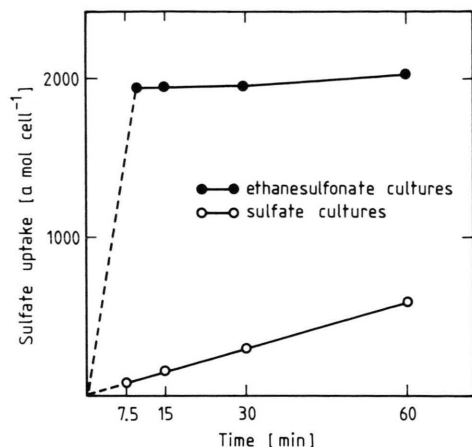| Cations [3 mM]	Sulfate uptake [% of control]
None	100
Li^+	100 ± 5
Na^+	98 ± 4
K^+	100 ± 5
Mg^{2+}	180 ± 17
Ca^{2+}	194 ± 14
Ba^{2+}	680 ± 157
Mn^{2+}	200 ± 13
Fe^{2+}	188 ± 29
Zn^{2+}	196 ± 19
Cd^{2+}	170 ± 17
Cu^{2+}	152 ± 10
Hg^{2+}	2 ± 1
Fe^{3+}	215 ± 24
Al^{3+}	225 ± 35

Fig. 6. Time dependence of enhanced sulfate uptake. Algae were grown for 5 days on 0.3 mM ethanesulfonate. For comparison the data for sulfate uptake in sulfate medium are included. Average of three replicates; average SE 14.6%.
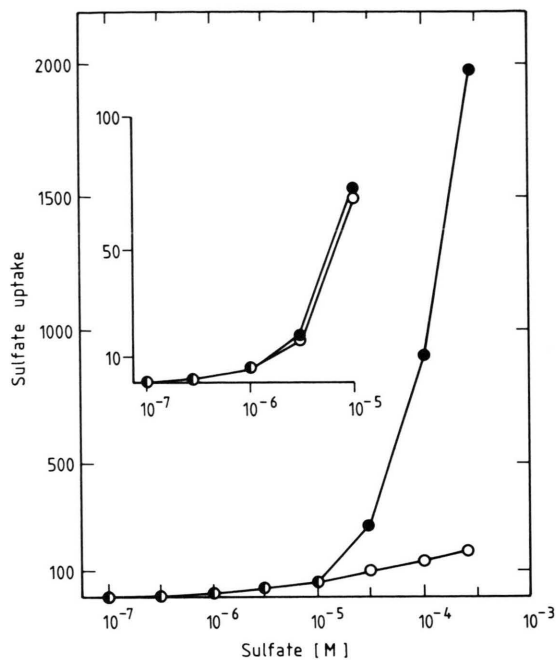

Fig. 7. Concentration dependence of enhanced sulfate uptake in S-starved algae in comparison to constitutive uptake in non-starved cultures. Uptake period 15 min. Average of two replicates; average SE 12.8%. O———O = sulfate culture; ●———● = ethanesulfonate culture.

Kinetics of enhanced sulfate uptake

The rate of sulfate uptake was determined in the concentration range from 1×10^{-7} to 3×10^{-4} M. No typical Michaelis-Menten hyperbolic curve was obtained plotting uptake velocity *versus* substrate concentration and no meaningful kinetic constants were obtained when the data were expressed in classical reciprocal plots. Comparing the concentration dependence of sulfate uptake in normal and sulfate starved cultures showed a clear separation point (critical concentration) around 0.01 mM sulfate (Fig. 7). At concentrations below 0.01 mM both uptake velocities were nearly identical and straight lines with similar K_m-values each were obtained using the Lineweaver-Burk plot. A K_m-value of 1.0×10^{-5} M for sulfate uptake could be determined (data not shown), comparable to the K_m of phase 1 for constitutive sulfate uptake (Table I).

At concentrations above 0.01 mM, sulfate uptake in S-starved cultures showed exceeding transport rates (Fig. 7), and the reciprocal plot revealed a concave upwards curve with negative values for K_m and V_{max}. Thus, data obtained at higher sulfate concentrations cannot be interpreted in terms of classical Michaelis-Menten kinetics. They must be composed of 2 or more kinetic systems as known from other transport processes in which an enhanced early uptake contributed significantly to total uptake [16].

Effect of pH on enhanced sulfate uptake

Sulfate uptake in starved cultures was hardly affected by the pH in the range of pH 5.0–9.0 (Fig. 8). A slight optimum around pH 9.0 might be seen in accordance with constitutive sulfate uptake in this alga (compare Fig. 1).
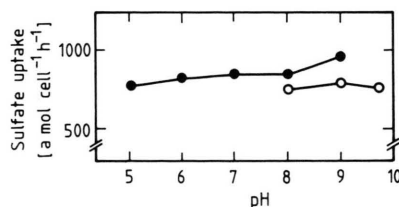

Fig. 8. pH-dependence of enhanced sulfate uptake. Ethanesulfonate grown algae were incubated in 0.01 M KH_2PO_4/K_2HPO_4 buffer (pH 5.0–9.0) or in 0.01 Tris/HCl buffer (pH 8.0–10.0). Uptake period 30 min. Average of two replicates; average SE 5.9%. ●———● = phosphate buffer; O———O = Tris-HCl buffer.
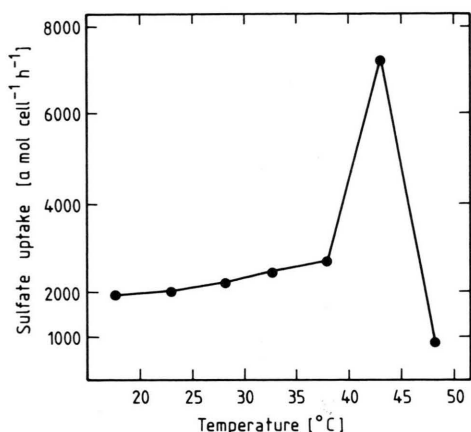
Fig. 9. Temperature dependence of enhanced sulfate uptake. Ethanesulfonate grown algae were suspended in prewarmed S-free medium and assayed for sulfate uptake after 15 min incubation. Average of two replicates; average SE 11.0%.

Fig. 10. Arrhenius plot of constitutive and enhanced sulfate uptake. The data of Fig. 2 and 9 were used.

Effect of temperature on enhanced sulfate uptake

The temperature optimum of 43 °C was well-marked (Fig. 9) and identical to the value determined in sulfate-grown cultures. The temperature dependence was greatly reduced to a Q_{10} of 1.2 between 18 °C and 38 °C, whereas the Q_{10} rose to 2.7 between 33 °C and 43 °C. The Arrhenius plot from 18 °C to 43 °C was biphasic with a discontinuity at 38 °C (Fig. 10). Both activation energies were calculated to 15.1 kJ/mol between 18−38 °C and to 163.0 kJ/mol above 38 °C. Arrhenius plots with an upward bend are unusual, since activation energies normally decrease at higher temperatures.

Substrate specificity of enhanced sulfate uptake

The specificity of the sulfate uptake system(s) involved was tested by competition experiments using group IV anions of the type XO_4^{2-} in a tenfold concentration as compared to sulfate. Inhibition of these analogues was comparable to their effects on constitutive sulfate uptake.

Discussion

The freshwater organism *Chlorella fusca* was grown under adequate mineral nutrition to avoid physiological side effects on sulfate uptake by starvation. These conditions were used to characterize sulfate tran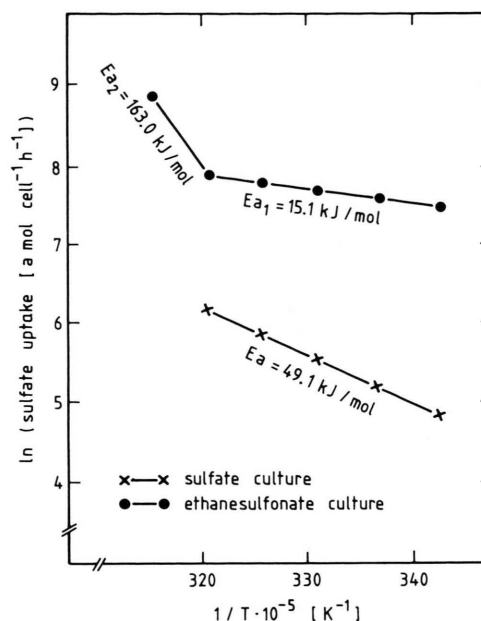sport as an active, multiphasic process with optima of 43 °C and pH 9.0 and a specificity for anions of the type XO_4^{2-} (part A).

Sulfate transport in non-deprived *Chlorella fusca* was multiphasic as in higher plant systems. Multiphasic uptake has been reported for roots and leaves of barley and for a variety of other plants [14, 17]. Singh and Paul [18] observed tetraphasic uptake of sulfate in excised maize roots, Shargool and Ngo [19] found biphasic uptake in excised roots of rape seedlings; whereas Jones and Smith [20] observed monophasic sulfate uptake into cultured tobacco cells. Also *Fucus serratus* showed a monophasic response to external sulfate in the concentration range 10^{-6} M to 10^{-2} M [7].

In the case of *Chlorella fusca*, sulfate uptake could be described by a single multiphasic isotherm having three phases. Each phase covered a limited concentration range and obeyed Michaelis-Menten kinetics (see [14]). The K_m of phase I 1.3×10^{-5} M and phase II 3.9×10^{-5} M were relatively high compared to other freshwater organism, *e.g. Chlorella pyrenoidosa* 1.2×10^{-6} M [5], *Synechococcus* 6301 7.5×10^{-7} [21] or *Spirulina platensis* 1×10^{-7} M [22]. However, sulfate concentrations in freshwater have been reported as $0.11 - 0.18$ mM [23]; thus the K_m of phase II

3.9×10^{-5} M is sufficient to allow adequate nutrition with sulfate.

Sulfate transport was highly dependent on temperature and pH in the assay, although sulfate represents a divalent anion throughout the whole range tested. The temperature optimum was about 43 °C; this value is markedly above the temperature tolerance of this strain. However, uptake of uracil and guanine in *Chlorella fusca* was also optimal around 40 °C [13, 24]. The activation energy between 18 °C and 43 °C was 49.1 kJ mol^{-1}, calculated by an Arrhenius plot for *Chlorella fusca*. This value seems to be related to the double negative charge involved, since the activation energy for uptake of electroneutral guanine is 35.5 kJ mol^{-1} [13], and the activation energy for uptake of the monovalent ethanesulfonate was 41.3 kJ mol^{-1} [12].

The pH-optimum of 9.0 for sulfate uptake in *Chlorella fusca* is similar to that of cyanobacteria [21, 22, 25], but unusually high compared with fungi or higher plants [2, 26, 27]. It may be adapted to the natural conditions of the environment, since *Chlorella fusca* is tolerant to pH-values between 3.5 and 10.5 during cultivation.

The high pH-optimum and the relatively low membrane potential of −46 mV in *Chlorella fusca* [28] do not support the idea of sulfate uptake *via* simple proton cotransport. Sulfate transport was further enhanced in the presence of divalent or trivalent cations in the medium found also for ethanesulfonate uptake in the same alga [12] and for sulfate uptake in fungi [2, 26]. Polyvalent cations could enhance uptake of anions *via* reduction of the surface potential [2], thus favouring the accumulation of anions at the binding site. However, one clear difference between *Chlorella fusca* and the fungal systems was obvious: None of the *Aspergillus* or *Penicillium* strains tested transported sulfate at measurable rates in the absence of polyvalent cations, whereas *Chlorella fusca* was able to do so [2, 26]. The "stimulatory" effect of Ba^{2+} was probably due to precipitation of Ba-[^{35}S]sulfate on the algal surface rather than to effects *via* surface potential, membrane potential or cell pH.

Sulfate uptake in *Chlorella fusca* is an energy-requiring process, as shown by the negative effects of various metabolic inhibitors (cyanide, azide, DCMU, DMBIB, CCCP, DCCD) and darkness. Sulfate uptake is active and occurs against a concentration gradient since the concentration of sulfate obtained by diffusion should not exceed 0.008 mM (see

Nernst equation: assuming a membrane potential of −46 mV, a charge of −2, and an external sulfate concentration of 0.3 mM). Since the sulfate level in normally grown cells of *Chlorella fusca* is about 1 mM [10], a 125-fold difference in concentration is achieved.

The existence of specific membrane-binding sites for sulfate is suggested by the inhibitory effect of the structural analogues chromate, selenate, sulfite and thiosulfate [5, 29, 30]. However, the sulfate permeases so far known differ in their affinity for molybdate and tungstate, both significantly larger anions than sulfate [29]. These two anions are not recognized by the sulfate permease of *Chlorella fusca*, whereas chromate and selenate strongly inhibit sulfate uptake. Obviously *Chlorella fusca* discriminates among anions of type XO_4^{2-} by the size of the molecule itself, as observed in *Monochrysis lutheri* [29] and *Fucus serratus* [7]. In contrast to these results, *Chlorella pyrenoidosa* [5], *Spirulina platensis* [22] or fungi [30] cannot discriminate between sulfate and MoO_4^{2-} or WO_4^{2-}.

Sulfite, thiosulfate and tetrathionate, which significantly inhibited sulfate uptake in *Chlorella fusca*, can also be seen as divalent analogues of sulfate. Especially thiosulfate was recognized as an alternative substrate of the sulfate permease in fungi [26, 30], in algae [5], and higher plants [27]. The monovalent anion methanesulfonate with structural relationship to thiosulfate was not inhibitory for sulfate uptake, demonstrating that the sulfate permease of *Chlorella fusca* has both size and charge recognition properties as known from other transport systems.

The inhibitory effect of cysteine and other mercaptides on sulfate uptake in *Chlorella fusca* may be explained as a chemical modification of the permease by SH-groups, regulating its state of activity [27]. Methionine, another important product of sulfate metabolism, did not inhibit sulfate transport. However, in contrast to higher plant systems, neither cyst(e)ine nor methionine could be identified as the main regulator for sulfate uptake in *Chlorella fusca* since only the size of the sulfate pool correlated with the actual transport rate [10].

Sulfate-starved cultures were obtained either by sulfur depletion or by adding ethanesulfonate, which allows growth under conditions of sulfate starvation [10]. Enhanced sulfate uptake could be measured using both growth conditions stated above. A time period of 15 or 30 min was chosen for determining

sulfate uptake, although later data showed that uptake was saturated within 7.5 min. Since the turnover of the sulfate pool is low during this time period, the data concerning the sulfate pool size are correct, however the actual uptake rates measured under sulfur limitation might be higher. Thus further experiments are necessary using a rapid test system for uptake rates within 1 min or less to obtain results measured under true kinetic conditions under sulfur limitation.

Enhanced sulfate uptake in sulfate-starved cultures differed significantly from constitutive sulfate transport in *Chlorella fusca:* 1) Time kinetics was not linear but showed an initial rapid phase followed by a lower steady state rate. 2) Uptake was neither multiphasic nor conformed to simple Michaelis-Menten kinetics. At higher sulfate concentrations, uptake was significantly enhanced resulting in negative values for K_m and V_{max} (see [31]). 3) Enhanced sulfate uptake was hardly affected by the pH in the range of pH 5.0–9.0. 4) Enhanced sulfate uptake was little affected by temperature in the range from 18 °C to 38 °C with a Q_{10} of 1.2 and an activation energy of 15.1 kJ mol^{-1}. The temperature optimum of 43 °C was similar to that of unstarved cultures. 5) The Arrhenius plot was biphasic with a bend at 38 °C. Above this temperature the activation energy increased tenfold to 163.0 kJ mol^{-1}. According to Dixon and Webb [32] we interpret this type of Arrhenius plot as a dissociation of the sulfate transport system into subunits at a critical temperature, parallel to a high temperature coefficient.

These data clearly demonstrate differences between sulfate uptake in normal and sulfate-starved cultures of *Chlorella fusca*. Thus, the common methodical "trick" to use conditions of sulfur starvation for the characterization of sulfate uptake systems might result in data not comparable with sulfate transport systems in non-induced organisms. A higher specific activity of [^{35}S]sulfate should be used instead of physiological starvation with significant changes in uptake rate and mode, even if this results in a greater adsorption or radioactivity to the cell surface.

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