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Sulphate Ion Influx and Efflux in *Hydrodictyon reticulatum*

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Abstract. Sulphate ion is accumulated in the cells of the alga *Hydrodictyon reticulatum* to a concentration approximately 30 mmol l^{-1}. The rate of SO_4^{2-} uptake is increased markedly in the light, however, the effect is observable only after the cells have been kept in the light for about one hour. Uptake of sulphate is strongly inhibited by phosphorylation uncouplers and also by DIDS. The two-phase sulphate uptake kinetics, reported earlier was confirmed in experiments in which competition with phosphate anion was tested. Phosphate competes with sulphate only in the range of higher substrate concentrations (from 0.2 mmol l^{-1}) and does not affect the system which works at low substrate concentrations. Efflux of SO_4^{2-} is very slow; the reason of the flux hindrance is not yet known.

Vastly different contents of sulphate anion have been reported in various algal species, ranging from more than 0.2 mol l^{-1} in *Desmarestia* (EPPLEY and BOVELL 1958, CLELAND *et al.* 1984) to only traces in *Nitellopsis* (ROBINSON 1969a). The uptake kinetics as influenced by inhibitors and light conditions were described in some detail for microalgae (*Chlorella*, *Scenedesmus*, see KYLIN 1967, VALLÉE and JEANJEAN 1968) and for *Chara* (ROBINSON 1969a,b). Light was found either to inhibit (*e.g.* in *Chara*) or to stimulate (*e.g.* in *Chlorella*) the uptake of sulphate. The diverse behaviour of the transport systems for sulphate ions in various algal species together with a lack of information about the efflux stimulated the experiments on the fresh water alga *Hydrodictyon reticulatum*, summarized in the present paper.

MATERIAL AND METHODS

All experiments were carried out with mature nets of the alga *Hydrodictyon reticulatum*, cultivated as described earlier (RYBOVÁ *et al.* 1987). Before experiments the algae were equilibrated in an artificial pond water containing sodium (1 mmol l^{-1}), potassium (0.1 mmol l^{-1}) and calcium (0.1 mmol l^{-1}) chlorides. Unless otherwise stated, the same medium with 0.1 mmol l^{-1} sodium sulphate labelled with ^{35}S was used in the experiments. When influx of SO_4^{2-} was measured, about 30 mg of algae were suspended in 1 ml of 0.01 mol l^{-1} H_2SO_4 for 4 days and the radioactivity was then estimated in 0.5 ml aliquots by a conventional scintillation method. In experiments where the efflux of SO_4^{2-} was estimated, about 200 mg of algal cells pre-

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incubated with radioactive sulphate for 10 days and then placed in nylon netting pouches were passed at definite time intervals through a succession of beakers containing the elution medium. Radioactivity was assessed in aliquots of the medium taken from the beakers and the portion remaining in the algae was estimated by the above procedure. All experiments were carried out at room temperature (approximately 20 °C).

Content of SO_4^{2-} was determined either as benzidine sulphate by the method described by DODGSON and SPENCER (1953) or by the more specific method of VICK (1975, *cf.* also LASS and ULLRICH-EBERIUS 1984). This is based on addition of Ba-chloranilate to samples and colorimetric estimation of free chloranilate after the insoluble $BaSO_4$ is precipitated and removed together with excess solid Ba-chloranilate. About 150 mg of algae were extracted in 4 ml water for 30 min on a boiling water bath. After adjusting the volume to its original level, 2 ml extract was transferred to a test-tube and 1 ml of 0.05 M phthalate buffer of pH 4 plus 2 ml of ethanol were added. After adjusting the pH of the solution to 4.0, 5 mg of Ba-chloranilate was added and the mixture was shaken overnight at room temperature. Solids were removed by centrifugation and the intensity of the pink colour measured at 530 nm on a DU8 Beckman spectrophotometer. Total content of sulphur in dry solids was estimated by the oxygen-flask combustion method.

RESULTS

First we measured the time dependence of sulphate uptake from a medium containing 0.5 mM SO_4^{2-} in the light. Fig. 1 shows that the substrate was taken up roughly linearly for the first 15 h and tended to be accumulated to

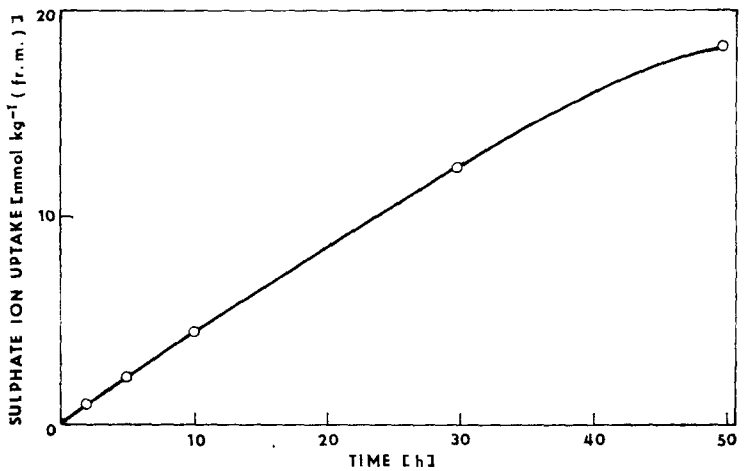

Fig. 1. Time dependence of sulphate ion uptake into the alga *Hydrodictyon reticulatum* in the light.

Abbreviations used: ATP = adenosine 5′—triphosphate; CCCP = carbonylcyanide *m*-chlorophenylhydrazone; DCMU = 3-(3,4-dichlorophenyl)1,1-dimethylurea; DIDS = 4,4′-diisothiocyanatostilbene 2,2′-disulphonic acid; DNP = 2,4-dinitrophenol; SITS = 4-acetamido-4′-isothiocyanatostilbene 2,2′-disulphonic acid; TRIS = tris(hydroxymethyl) aminomethane.

TABLE 1
Sulphate concentration in the cells of *Hydrodictyon reticulatum*

c_o [mmol l^{-1}]	c_i [mmol l^{-1} cell water]	
0.1	27.4 ± 1.5	(n = 6)
0.5	30.7 ± 1.9*	(n = 5)
1.0	32.8 ± 0.8	(n = 6)
5.0	33.8 ± 2.4	(n = 6)

* determined as benzidine sulphate

a concentration well above 20 mmol kg^{-1} of algal fresh mass. One may argue that the value obtained after 50 h of continuous light might be afflicted by an error caused by sulphate efflux, but, as demonstrated later, this would be of no significance.

Determinations of free sulphate estimated either as benzidine sulphate or as chloranilate equivalents demonstrated that its concentration in the cells was indeed some 30 mmol kg^{-1} intracellular water. The internal concentration was shown to depend to a certain degree on the concentration of sulphate outside the cells (Table 1). The total sulphur present in the cells was on average 1.18 % of dry solids; with 9.53 kg of water per kg dry solids the free sulphate thus represents about 78 % of the cellular S; hence any error of our results due to sulphate metabolism in the course of the relatively short experimental periods is likely to be negligible.

The uptake of sulphate ions is profoundly affected by the light conditions. The response to changes in light conditions, however, is, not immediate but starts only after a certain time lag (Fig. 2). Especially with light-pretreated cells the higher absorption rate in the dark persists for quite a long period. A sigmoid curve is obtained in the light after 12-h dark pretreatment pointing to the fact that an intensively transporting system for sulphate is developed in the light during the first hour. After a 10-h incubation 7 times more sulphate entered light-pretreated cells in the light when compared with dark-pretreated cells in the dark. When expressed as fluxes, the light unidirectional influx was 0.48 pmol cm^{-2} s^{-1} whereas the initial dark influx was 0.16 pmol cm^{-2} s^{-1}.

The concentration dependence of sulphate uptake under light or dark conditions studied in the range of 0.01 to 1 mM revealed that the kinetics does not correspond to a simple uptake isotherm. Instead, as previously demonstrated in more detail for the dependence in the light (RYBOVÁ *et al.* 1982), the kinetics corresponds to a classical Michaelis-Menten hyperbola only at higher substrate concentrations (from 0.25 to 1 mM) and what remains when the hyperbola is subtracted from experimental points can be expressed as a function $k_1 c \exp(k_2 c)$, presented by dashed line in Fig. 3. The latter curve fits a mechanism that is active only at very low concentrations and has a very high affinity to the substrate. Both mechanisms are markedly affected by the light conditions. A similar atypical "hump" curve was described in the alga *Valonia* (MITA *et al.* 1984). At higher substrate concentrations the J_{max} changed from 0.76 pmol cm^{-2} s^{-1} in the light to 0.12

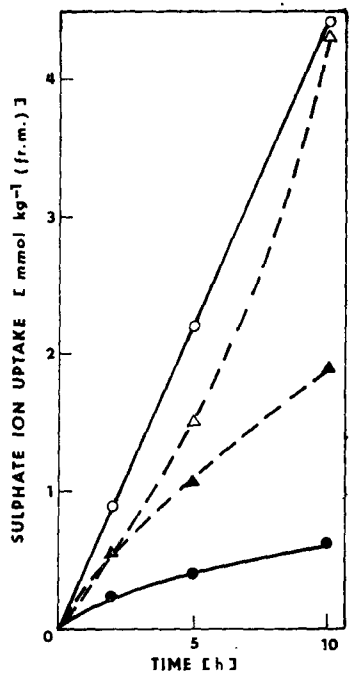

Fig. 2. Effect of light conditions on sulphate ion uptake by *Hydrodictyon reticulatum*. — (Empty circles, full line — light, preincubation in the light; empty circles, dashed line — light, preincubation in the dark; full circles, full line — dark, preincubation in the dark; full circles, dashed line — dark, preincubation in the light).

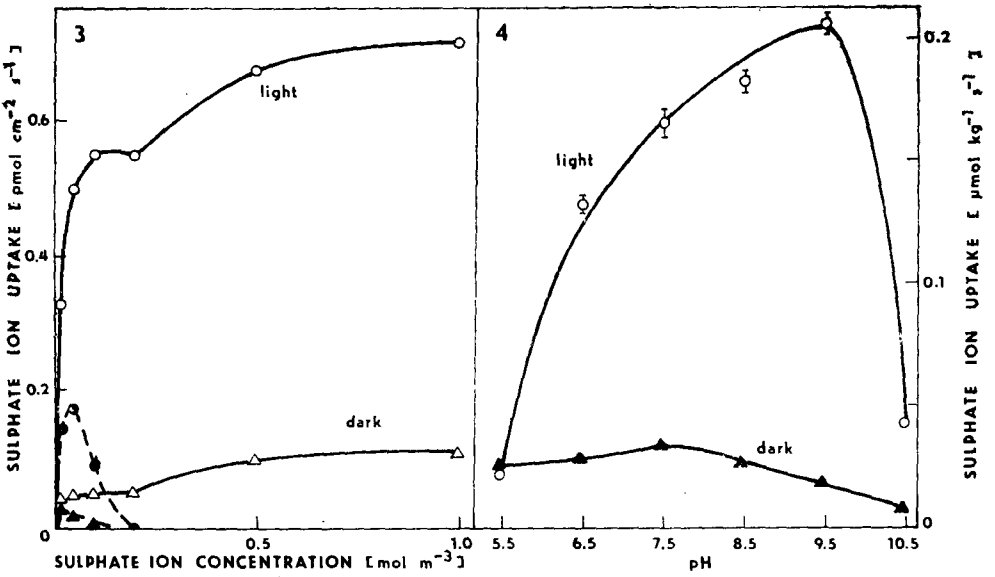

Fig. 3. Initial rate of SO_4^{2-} uptake as dependent on sulphate ion concentration in the light and dark. (Empty circles — light, empty triangles — dark, full circles and triangles — differences between the appropriate hyperbolas and experimental points).

Fig. 4. Effect of pH in the medium on SO_4^{2-} uptake into *Hydrodictyon reticulatum* in the light and dark. (Circles — light, triangles — dark). Medium buffered with mixtures of phtallic acid (5 mmol l^{-1}) and triethanolamine (10 mmol l^{-1}) in artificial pond water.

pmol cm^{-2} s^{-1} in the dark and the transport constant $K_T = 6 . 10^{-5}$ M increased about twice.

The stimulating influence of photosynthetic activity on the uptake of sulphate is also obvious from the pH dependence of this transport process. Fig. 4 shows a pronounced effect of external pH on SO_4^{2-} uptake in the light with a maximum at pH about 9. The rate of photosynthesis appears to have a maximum near this value in the external medium (RYBOVÁ and JANÁČEK 1982). In the dark, the external pH does not have such a marked effect on sulphate uptake: between 5.5 and 8.5 only an indistinct maximum at pH 7.5 may be observed and there is an evident decrease in the transport activity at pH's higher than 9.5.

TABLE 2

Effect of uncouplers and DCMU on the SO_4^{2-} influx (in mmol kg^{-1} fresh mass)

Duration of incubation [h]	Controls	$5 . 10^{-7}$ M CCCP	10^{-5} M DNP	10^{-7} M DCMU	$5 . 10^{-7}$ M DCMU
		in the light			
5	2.62 ± 0.3	2.01 ± 0.05	0.12 ± 0.01	3.30 ± 0.15	2.73 ± 0.2
10	5.32 ± 0.5	2.48 ± 0.2	0.09 ± 0.1	2.84 ± 0.3	3.24 ± 0.4
		in the dark			
2	0.72 ± 0.01	0.29 ± 0.03	0.28 ± 0.02	–	–
5	1.09 ± 0.1	0.26 ± 0.03	0.42 ± 0.02	–	–

/ Averages of 6 determinations).

To find out what are the energy sources for sulphate uptake and whether the transport mechanism may be directly attacked, several metabolic and ion channel inhibitors were used. Table 2 shows that uncouplers of phosphorylation (or proton conductors) such as DNP or CCCP markedly inhibit SO_4^{2-} irrespective of the light conditions. In the light the sulphate uptake decreased to 4.6 % of the controls in the presence of 10^{-5} M DNP and to 76.7 % in the presence of $5 . 10^{-7}$ M CCCP after a 5-h incubation. In the dark, the two inhibitors reduce the transport to some 40 % of the controls after a 2-h incubation. On the other hand, DCMU, a specific inhibitor of the second photosystem, had, at 10^{-7} M concentration, rather a stimulatory effect on the light uptake in the first five hours. However, after a prolonged, 10-h incubation it reduced the uptake by 47 % which points to the fact that the enhanced uptake rate of sulphate in the light requires the maintenance of the integrity of the photosynthetic system.

Table 3 compares the effects of SITS (4-acetamido-4′-isothocyanatostilbene-2,2′-disulphonate) and DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonate), specific inhibitors of anion transport. The latter inhibitor is obviously more potent in *Hydrodictyon* cells, as only about 10.5 % of sulphate is taken up after a 5-h incubation and 4.9 % after a 10-h incubation in its presence as compared with the controls, whereas SITS at the same con-

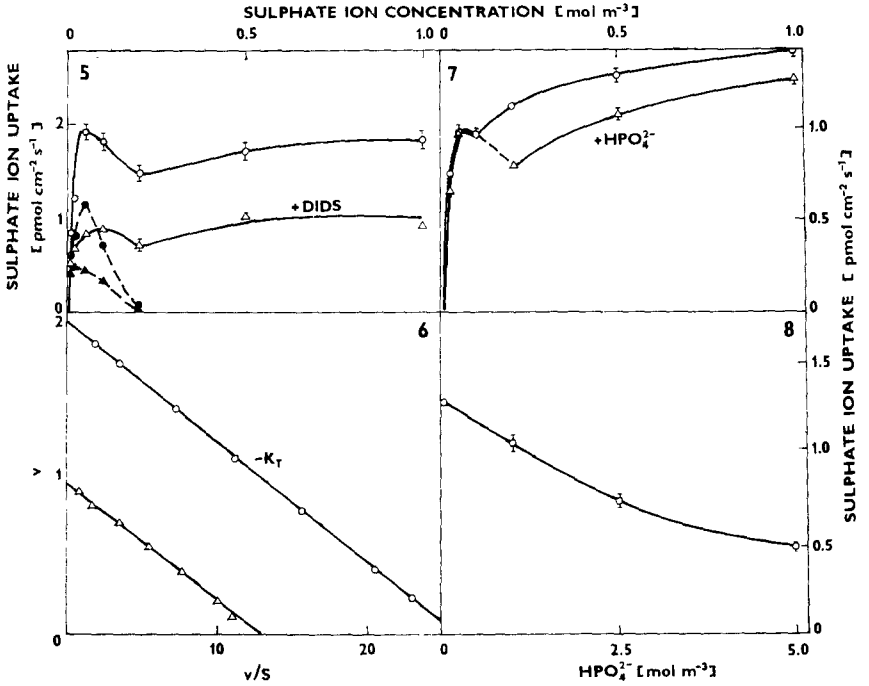

Fig. 5. Effect of DIDS on the concentration dependence of SO_4^{2-} inflow into *Hydrodictyon reticulatum*. (Empty circles — controls, empty triangles — 10^{-4} DIDS, full circles and triangles — differences between the appropriate hyperbolas and experimental points).
Fig. 6. Woolf-Hofstee plot of the sulphate uptake kinetics of control and DIDS inhibited algal cells. (Circles — controls, triangles — DIDS).
Fig. 7. Concentration dependence of SO_4^{2-} inflow into *Hydrodictyon reticulatum* in the presence of HPO_4^{2-} (1 mmol l^{-1}). (Circles — controls, triangles — HPO^{2-}_4 added).
Fig. 8. Effect of HPO_4^{2-} on sulphate ion inflow into the alga *Hydrodictyon reticulatum*. (Concentration of sulphate anion 0.5 mmol l^{-1}).

centration does not influence the uptake after 5 h and inhibits it to 57.8 % after 10 h. As shown in Fig. 5, DIDS (at 10^{-4} M concentration) inhibits sulphate ion uptake in the broad range of sulphate concentrations tested (*i.e.* from 10^{-5} up to 10^{-3} M), affecting both uptake systems, and behaves as a classical noncompetitive inhibitor influencing only J_{max} (from 1.96 in the controls to 0.95 in the presence of DIDS) but not the K_T (Fig. 6).

One may expect that of the metabolically important anions phosphate may share some transport step with sulphate and hence compete with it. We therefore tested the influence of divalent monohydrogen phosphate anion on sulphate uptake. To prevent pH changes close to the external cell surface, the medium was buffered with 5 mM TRIS and the pH value adjusted to 9.0 with hydrochloric acid. As shown in Fig. 7, the phosphate anion does not affect sulphate uptake at low substrate concentrations. However, at concentrations from 0.2 to 1 mM, where the Michaelis-Menten type of kinetics appears to be operative, phosphate exerts an inhibitory effect. The control value of K_T in this series of experiments was somewhat higher (10^{-4} M) when compared to values obtained earlier, possibly due to the buffering

TABLE 3
The effect of transport inhibitors on SO_4^{2-} influx [mmol kg^{-1} fresh mass] in the light

Duration of incubation [h]	Control	$5 . 10^{-4}$ M SITS	10^{-4} M DIDS	$5 . 10^{-4}$ M DIDS
5	4.3 ± 0.2	4.46 ± 0.2	1.41 ± 0.04	0.45 ± 0.02
10	8.18 ± 0.8	4.73 ± 0.6	2.53 ± 0.12	0.97 ± 0.2

(Averages of 6 determinations).

of the medium. In the presence of phosphate, K_T was doubled, whereas J_{max} remained at 1.5 pmol cm^{-2} s^{-1}. The phosphate inhibition has a competitive character (*cf.* also Fig. 8).

In contrast to the relatively high initial rate of sulphate influx the efflux of this anion from cells containing about 30 mM SO_4^{2-} into a medium with 0.1 mM SO_4^{2-} was practically negligible. Compartmental analysis of the efflux curve (Fig. 9) showed that under light conditions in unbuffered media (which rather corresponds to a slightly buffered alkaline medium as the alga alkalizes its surroundings strongly in the light), 96.3 % of the activity is exchanged with a half-time which cannot be assessed with certainty but which exceeds 3500 h. The compartment exchanging sulphate with this negligible rate appears to correspond to vacuole, as in *Hydrodictyon* this organelle takes up about 90 % of the total cell volume (NEŠPŮRKOVÁ 1983). About 1.5 % of the activity is exchanged with a half-time of 2.5 h; the corresponding compartment may represent the cytoplasmic layer. The last fraction that exchanges very rapidly includes the free space in the cell wall and probably the surface contamination. The dark efflux was larger than the light one. This difference, as will be seen below, may be brought about by the rather acidic pH at the cell surface in the dark.

Decreasing the pH in the outer medium increases somewhat the efflux from the cells (Fig. 10) as if supressing the dissociation of acidic groups in

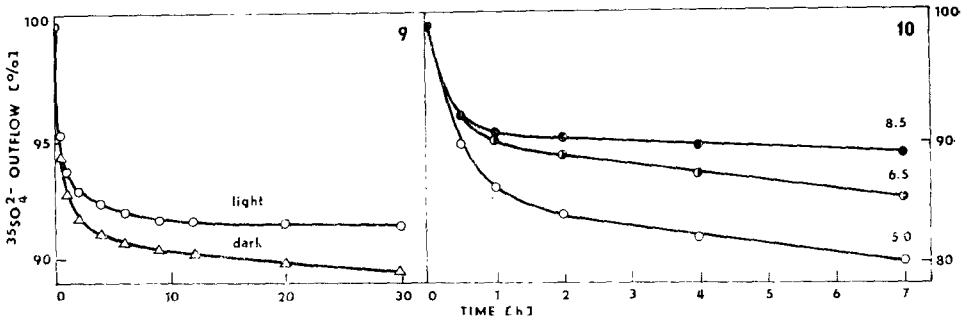

Fig. 9. $^{35}SO_4^{2-}$ outflow [per cent of initial radioactivity] from the alga *Hydrodictyon reticulatum*. (Circles — light, triangles — dark).
Fig. 10. Effect of pH of the medium on $^{35}SO_4^{2-}$ outflow [per cent of initial radioactivity] from the alga *Hyrdodictyon reticulatum* in the light.

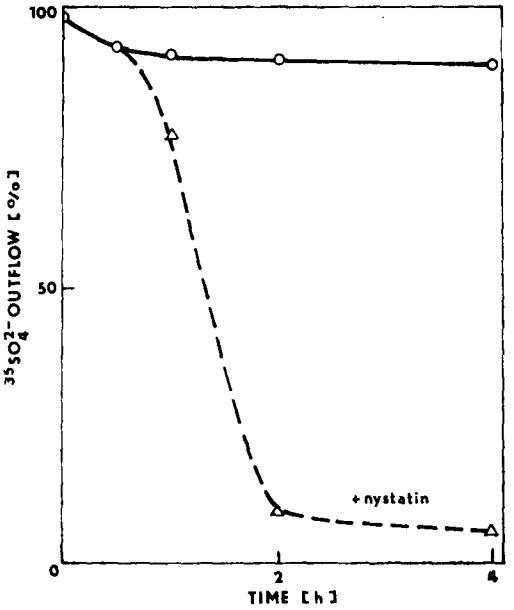

Fig. 11. $^{35}SO_4^{2-}$ outflow from algae after addition of 0.01 % nystatin (full line — control, dashed line — nystatin).

the membrane was playing a positive role in the sulphate outflow. Metabolic inhibitors (DNP, CCCP) did not alter the efflux rate of SO_4^{2-}. Only 10^{-4} M Tl^+ exerted an effect comparable to decreasing the outer pH to about 7. After permeabilization of the plasma membrane and tonoplast with nystatin the sulphate anion leaves the cell rapidly (Fig. 11).

DISCUSSION

The relatively high level to which free sulphate is accumulated in *Hydrodictyon reticulatum* makes this alga a suitable object for kinetic transport studies of SO_4^{2-} anions. The intracellular concentration of sulphate was found to be approximately 30 mM and thus it appears that the divalent sulphate, together with 75 mM chloride anion, balances, presumably in the vacuole, the most abundant cation — potassium, the concentration of which is 139 mM on average.

Sulphate is transported into the cells against a large concentration gradient and, as a bivalent anion, it also performs considerable electrical work because the transmembrane potential in *Hydrodictyon reticulatum* is approximately 100 mV, inside negative (Rybová *et al.* 1987). Hence it may be classified among actively transported ionic species in *Hydrodictyon*. The kinetics of absorption and release of sulphate, the effect of light as well as the action of inhibitors differ in many respects from those observed with non-metabolizable chloride (Rybová *et al.* 1972). Whereas, *e.g.*, the uptake of chloride anions is changed within several minutes in response to newly established light conditions, sulphate uptake reacts only after a considerable delay. It appears that the intensive light-stimulated uptake of SO_4^{2-} is controlled

by a system which is set up in the light and a certain period of time is necessary for this. Once developed, the system maintains the ability to transport sulphate for more than an hour in the dark. The whole character of the light on SO_4^{2-} uptake has much in common with that observed in *Chlorella* cells by VALÉE and JEANJEAN (1968). In algae, light-stimulated transport was reported also for *Scenedesmus* (KYLIN 1964) and *Porphyridium* (RAMUS and GROVES 1972); on the other hand, in *Chara corallina* (*australis*) a light inhibition of sulphate transport was observed, whereas in *Nitella translucens* the changing of light conditions did not have any apparent effect (ROBINSON 1969a).

The preferential utilization of ATP derived from respiration as a source of energy for sulphate uptake appears to explain the higher dark accumulation of SO_4^{2-} in Characean cells; this, however, cannot apply to *Hydrodictyon reticulatum* with its light-stimulated sulphate uptake. Experiments with phosphorylation uncouplers showed that even if ATP serves most probably as an energy donor also in this organism (alternatively, proton gradient may be involved as, *e.g.*, in *Lemna* — LASS and ULLRICH-EBERIUS 1984) in the light, the ATP is derived in the process of photosynthetic phosphorylation. In contrast to the transport of some other anions, such as Cl^-, NO_3^- or HCO_3^-, DCMU at $5 \cdot 10^{-7}$ M concentration does not affect the transport of sulphate during the first 5 h and hence the functioning of the second photosystem is not an indispensable condition for sulphate uptake. At 10^{-7} M concentration DCMU exhibits a stimulating action, as if more energy were available for sulphate transport. It is only after a longer time period (between 5 and 10 h) that the presence of the inhibitor reduces the SO_4^{2-} uptake which may be due to the fact that prolonged inhibition of the PS II leads finally to a decrease in the content of the photoproduced ATP. Alternatively, one may expect that the control over the plasmalemma transport would reflect the capability of sulphate metabolism in the chloroplasts. It was demonstrated (ZIEGLER and HAMPP 1977) that DCMU inhibits sulphate uptake across the outer chloroplast membrane in spinach isolated particles together with its reduction and final appearance in the form of -SH groups.

The pH dependence of SO_4^{2-} uptake in the light with its maximum in the region of maximum photosynthesis (RYBOVÁ and JANÁČEK 1982, *cf.* also RAVEN 1968 for *Hydrodictyon africanum*) demonstrates most probably a close connection between sulphate absorption and photosynthesis. The curve obtained by plotting the dark SO_4^{2-} uptake against external pH coincides well with the dependence described for *Chlorella vulgaris* (SPEDDING *et al.* 1980) where a practically constant uptake rate of sulphate anion between pH 4.5 and 7.5 was found. These results confirm the view that the components of the transport mechanism proper are (at least in the dark) not sensitive to changes in external pH.

The results obtained on investigating the concentration dependence of sulphate uptake, which showed that there are two uptake mechanisms, have been summarized above. The results obtained by analyzing the competition of DIDS and HPO_4^{2-} with SO_4^{2-} for uptake appear to support the existence of two independent transport mechanisms for sulphate.

The two systems are strongly inhibited by DIDS, a specific inhibitor of anionic channels (see, *e.g.*, CABANTCHIK and ROTHSTEIN 1972). Sulphate transporting mechanisms of *Hydrodictyon reticulatum* are more sensitive to

this inhibitor than to its analogue SITS, and, moreover, DIDS appears to be a more potent inhibitor of transmembrane movement of sulphates than of chlorides (RYBOVÁ *et al.* 1984). This is in contrast to animal cells, *e.g.* red blood cells, where DIDS affects most strongly the transport of chloride anions.

Phosphate anion, on the other hand, competes with sulphate for entry in a higher concentration range where a normal Michaelis-Menten type of kinetics is operative. No competition was observed at low substrate concentrations (up to 0.1 mM) where an intensive, energy requiring system with a high affinity for SO_4^{2-} is at work. The physiological role of this latter system, the activity of which disappears at about 0.2 mM sulphate in the outer medium, appears to be to accumulate this substrate from sulphate-poor media. The system not only resists competition with a foreign substrate, but, moreover, helps to overcome the competition effect of phosphate on the second system. Assuming that the second, low affinity system already starts to work at low concentrations, the decrease of uptake due to its inhibition by competing HPO_4^{2-} ions should be noticeable also at lower substrate concentrations, but this is not so, as shown in Fig. 7. A classical two-phase isotherm kinetics, described by two Michaelis-Menten functions without a common origin, might explain this without difficulty, but our attempts to describe mathematically the first, high-affinity system as a hyperbola failed (RYBOVÁ *et al.* 1982).

For plant as well as for animal cells diverse results were reported concerning the question of competition between the transport of sulphate and phosphate anions. The degree of specificity of the binding proteins and channel system seems to differ between species, organs or even intracellular membraneous structures. Nevertheless, at least for plant mitochondria (ABOU-KHALIL and HANSON 1979) and chloroplasts (HAMPP and ZIEGLER 1977) one may hold for sure that sulphate ions enter these organelles *via* a phosphate translocator. Our results show that at higher sulphate concentrations a transport step is shared by HPO_4^{2-} and SO_4^{2-}.

The backflow of labelled sulphate to the medium, whether in the light or in the dark, is negligible. It has been observed with other algal species that the sulphate, once accumulated in the cells, is released only very slowly. Trapping of SO_4^{2-} in the acid vacuolar sap, independent of metabolic energy, takes place in *Desmarestia*, where sulphate is the accompanying anion to protons (CLELAND *et al.* 1984). In the rhizoids of *Chara* (SCHRÖTER *et al.* 1975) insoluble sediments with barium salts, the so-called statoliths, are formed inside the cells. The low concentrations of dissolved SO_4^{2-} may explain the low efflux rate, but no precipitates were detectable in *Hydrodictyon*. Compartmental analysis of the efflux curves in *Hydrodictyon reticulatum* led us to the conclusion that sulphate anion is accumulated in the vacuole. The mechanism responsible for the effective impermeability of the tonoplast to the outwardly directed movement of sulphate ion is not yet known. Nevertheless, if it is borne in mind that one of the main physiological roles of the vacuole is to serve as a storage organ one has to admit that it is an energy saving mechanism.

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