Uptake and Efflux of [^{35}S]Sulfite by Protoplasts and their Chloroplasts of Oat (*Avena sativa* L.)

Z. Miszalski and H. Ziegler

Lehrstuhl für Botanik, Technische Universität München, D-8000 München 2, Bundesrepublik Deutschland

Z. Naturforsch. **44c,** 509–513 (1989); received February 13, 1989

Dedicated to Professor Achim Trebst on the occasion of his 60th birthday

Avena sativa, Chloroplasts, Protoplasts, Sulfite Efflux, Sulfite Uptake

Processes of ^{35}S-labelled sulfite uptake and efflux by oat protoplasts and their chloroplasts are very rapid: during the first minutes (or perhaps even seconds) after sulfite supply a state of equilibrium is established between the protoplasts and their chloroplasts and the incubation medium. Bicarbonate concentrations ranging from 1 to 5 mM in the medium had no significant effect on sulfite uptake. At pH 7.0 sulfite uptake is much greater than at pH 7.6. Light of low intensity (9 W m^{-2}) had no significant effect on this uptake. The ^{35}S concentration in chloroplasts was always much higher than in the whole protoplasts. One hour's incubation at sulfite concentrations of 0.1 and 0.5 mM in the medium, concentrations slightly stimulating or inhibiting photosynthesis, results in sulfite concentrations of 0.004 to 0.060 mM in protoplasts and of 0.020 to 0.230 mM in chloroplasts. At a sulfite concentration of 10.0 mM in the medium, which inhibits photosynthesis, these values are 0.120 to 0.551 and 2.045 to 3.531, respectively.

Introduction

Photosynthetically active protoplasts in liquid suspension are an useful material for studying the response of mesophyll cells to SO_2 while avoiding the problems caused by the variable effects of SO_2 on stomatal diffusion resistance [1–3]. With the technique of rapid protoplast fractionation [4] it is possible to determine the uptake of sulfur compounds into the cells and their intracellular distribution and to correlate the specific effect, *e.g.* on photosynthetic activity, with defined concentrations. Our earlier work [2] demonstrated that the sensitivity of isolated oat protoplasts to sulfite depended mainly on $NaHCO_3$ concentration and the pH value of the medium.

The aim of this investigation was to proof whether these factors affected the amount of sulfur compounds penetrating into protoplasts and their chloroplasts. Sulfite concentrations in the experiments were adjusted so as to exert either a positive or a negative effect on the photosynthesis rate.

Abbreviations: BSA, Albumin bovine; HEPES, N-2-hydroxyethyl-piperazine-N′-2-ethane sulfonic acid; MES, 2/N-morpholino-ethane sulfonic acid; tricine, N-tris-hydroxymethyl-methyl glycine.

Reprint requests to Dr. Z. Miszalski. Permanent address: Dept. of Plant Physiology, Polish Academy of Sciences, 31-016 Kraków, ul. Sławkowska 17, Poland.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/89/0500–0509 $ 01.30/0

Material and Methods

Material

Seedlings of *Avena sativa* L. *cv.* Arnold and *cv.* Pirolle were grown on moist peat at 26 °C and 80% relative humidity for five days in darkness, than for two to three days in a greenhouse. When natural light was insufficient in the last stage of growth, plants were additionally illuminated (Osram HQLS 400 W lamp, 9 W m^{-2} at seedling level).

Isolation of protoplasts

Procedures for protoplast isolation were essentially according to [4] and [5]. Strips of leaves (*ca.* 1 mm wide) weighing *ca.* 10 g were incubated for 2.5 h in 2% cellulosin (from Calbiochem, Basel, Frankfurt), 0.5% BSA and 5 mM MES-KOH adjusted to pH 5.6, at 30 °C. The protoplast suspension was centrifuged, resuspended and purified on a sucrose-mannitol gradient ("sucrose": 0.5 M sucrose plus 1 mM $CaCl_2$; "mannitol": 0.5 M mannitol plus 1 mM $CaCl_2$ and 5 mM MES-KOH, pH 6.0). Protoplasts banded at the interface were separated and washed with the medium, also used for incubation, composed of 0.5 M sorbitol, 7.5 mM $CaCl_2$ and 100 mM tricine adjusted with HCl to pH 7.0 or 7.6, respectively, and $NaHCO_3$ at concentrations from 1 to 10 mM. The density of the suspension was adjusted to about 5×10^6 cells in 1 ml.

Incubation of protoplasts

The effect of Na_2SO_3 on the sulfur concentration inside the protoplasts and their chloroplasts was determined in protoplast suspensions after incubation in 0.1, 0.5, or 10.0 mM sulfite solutions labelled with $Na_2{}^{35}SO_3$ (74 kBq) for 1 h in light (9 W m^{-2}) or in darkness. pH was either 7.0 or 7.6. Incubation was in the presence of 1, 5 or 10 mM $NaHCO_3$. To check the quality of the isolated protoplasts evolution or uptake of O_2 in light was measured with a Clark-type oxygen electrode at 25 °C with 5 mM $NaHCO_3$. The light source was a 250 W projector, light intensity at the surface of the vessel used for measurements was about 300 W m^{-2}. The chlorophyll content was measured according to [6]. Only protoplasts with intact photosynthesis (110 μmol O_2 h^{-1} mg^{-1} of chlorophyll at pH 7.6 and 5 mM $NaHCO_3$ and about 100 μmol O_2 h^{-1} mg^{-1} of chlorophyll at pH 7.0 and 5 mM $NaHCO_3$) were used in the experiments.

Washing of protoplasts

In some experimental series protoplasts after incubation were centrifuged for 1 min at $300 \times g$, the residue was suspended in 20 ml of sulfite-free incubation medium and the density of protoplasts in the suspension was adjusted to the same level as at the beginning of incubation. Protoplasts were separated 5, 30 and 60 min after washing.

Fractionation of protoplasts

At the end of incubation protoplasts were filtered and simultaneously centrifuged in darkness. For this purpose 50 μl of protoplast suspension were pipetted into 400 μl tubes, some with and others without a 20 μm mesh net, containing a number of hydrophilic-hydrophobic layers [4, 7].

Tubes for fractionating protoplasts were prepared in two ways (Fig. 1), some to fractionate protoplasts and others to separate whole and damaged protoplasts. The integrated system of homogenating and fractionating protoplasts was used to obtain three fractions (chloroplasts, mitochondria and the remaining parts of cells), which were afterwards metabolically inactivated in a few seconds. Procedures for correction adjustment for cross contamination of the different fractions were according to [8] and [9]. Tubes equipped with a net were kept at 4–8 °C and those without at room temperature. After the transfer of protoplasts each tube was closed with a punctured cape and 10 μl of 0.5 M Tris-HCl buffer (pH 7.6) containing 1 M KCl and 50 mM $MgCl_2$ were placed in its hollow. All tubes (5 of each kind) were then centrifuged at 4–8 °C during 60 sec, at $12{,}000 \times g$ (microfuge B, Beckman small head). Immediately after centrifuging tubes were frozen in liquid nitrogen, sliced and covered with 0.5 ml of *aqua dest.* and 5 ml of scintillant (Quickszint 402, Zinsser). Radioactivity was measured 12 h later and than quenching was measured according to [10].

In every experimental series in addition to the fractionation of cells other tubes were prepared to determine quantitatively the content of cell organellae in each of the fractions. For this purpose after fractionation tubes were cooled in ice, the fractions were dissolved in a buffer (HEPES-KOH 50 mM, pH 7.6, 8 mM $MgCl_2$) and a part of the solution was used to measure the activity of marker enzymes, fumarase for mitochondria and NADP-dependent triose-P-dehydrogenase for chloroplasts. Fumarase (EC 4.2.1.2) was measured according to [11] using 50 μM tricine buffer, pH 8.4, starting the reaction by

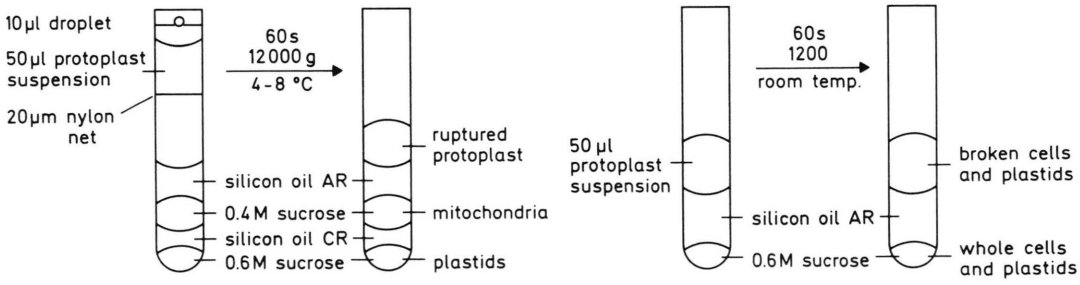

Fig. 1. Schematic presentation of the procedures of homogenization and fractionation of oat protoplasts (*cf.* [8]). Protoplast forced through a nylon net. The homogenate thus obtained is then fractionated on a density gradient formed by alternate layers of silicon oil (CR: density 1.070 g/ml at 25 °C and AR: density 1.030 g/ml at 25 °C) and sucrose solution (0.6 M and 0.,4 M). In tubes without a net undamaged protoplasts or chloroplasts are separated from the damaged ones by filtration through silicon oil (AR: density 1.028 g/ml at 25 °C).

adding L-malate up to the concentration of 10 mM and measuring the reduction of NAD at 240 nm. The activity of NADP-dependent triose-P-dehydrogenase (EC 1.2.1.9) was determined from the drop of absorption at 340 nm caused by NADPH oxidation by 3-phosphoglyceric acid [12].

With the data on the volume of chloroplasts and other cell organellae reported by [9] and the results of our own research it was possible to calculate the sulfite concentration in the protoplasts and chloroplasts used in the experiments. In order to determine the amount of radioactive sulfur taken up from the incubation medium and passed with the protoplasts and chloroplasts fractions through the hydrophilic-hydrophobic layers experiments were carried out with ^{14}C-labelled sucrose, which was added immediately before fractionation. The actual amount of ^{35}S was then obtained by reducing the previously calculated amount in the same proportion as the percentage of labelled sucrose that passed with the filtrate through the silicon layers.

Significance of results

Every experiment was made with five parallel samples for every set of the experimental conditions, and the experiment was repeated at least three times.

Results

No relationship was found between the amount of ^{35}S uptake and duration of incubation, which lasted 5, 30 and 60 min, presumably because the process is very rapid. Owing to technical problems the separation of protoplasts immediately after the beginning of incubation proved unfeasible. All other experiments were carried out after 1 h of incubation. It was not possible to determine the [^{35}S]sulfite concentration in the mitochondrial fraction probably because of the rest of incubation medium forcing through the AR-silicon oil layer.

To determine the rate of ^{35}S efflux from protoplasts they were submitted to the washing procedure after 1 h of incubation. As can be seen in Fig. 2, ^{35}S concentration (expressed as sulfite concentration) in protoplasts and chloroplasts dropped sharply during the first five minutes of washing, then the rate of the drop declined. After 1 h of washing the ^{35}S content was about 25 to 30% of the original content. Throughout the experiments the ^{35}S concentration in chloroplasts was 3 to 4 times higher than in whole protoplasts. The ^{35}S efflux rate from protoplasts was insignificantly higher than from chloroplasts.

The effects of the pH value and $NaHCO_3$ concentration in the medium on the uptake of ^{35}S were examined after one hour's incubation in the presence of 0.1 mM sulfite. The results illustrated in Fig. 3 indicate that a change of $NaHCO_3$ concentration from 1 to

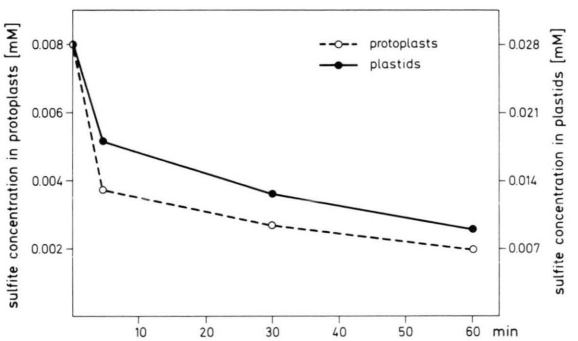

Fig. 2. Time-dependent changes of sulfite efflux rate from protoplasts and chloroplasts from oat to a sulfite-free medium. Protoplasts were previously incubated for 1 h in darkness in a medium containing 0.1 mM [^{35}S]sulfite and 1 mM $NaHCO_3$ at pH 7.0.

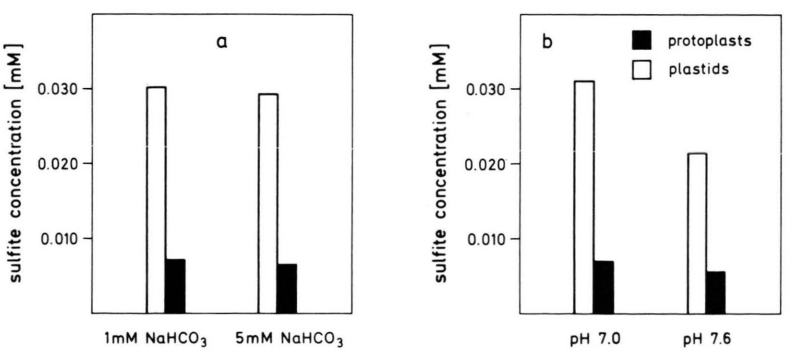

Fig. 3. Sulfite concentration in protoplasts and chloroplasts after 1 h incubation of protoplasts in darkness with 0.1 mM sulfite; a: media containing 1 mM or 5 mM $NaHCO_3$, pH 7.0; b: media of pH 7.0 or 7.6. The data of b are mean values of measurements made at $NaHCO_3$ concentrations of 1, 5 and 10 mM.

5 mM at pH 7.0 had only a slight reducing effect on the levels of labelled sulfur in protoplasts and chloroplasts. At all $NaHCO_3$ concentrations the uptake of ^{35}S at pH 7.6 was lower than at pH 7.0, in protoplasts as well as in chloroplasts. In view of these results the next series of determinations was made in a medium containing 5 mM $NaHCO_3$ at pH 7.6, as conditions inhibiting ^{35}S uptake, and 1 mM $NaHCO_3$ at pH 7.0, as conditions stimulating the process. The results are listed in Table I. Measurements were made at three sulfite concentrations: 0.1, 0.5 and 10.0 mM, the first two being concentrations that either slightly activated or slightly inhibited $^{14}CO_2$ uptake and the last strongly inhibiting this uptake [13]. For all three sulfite concentrations the ^{35}S uptake was measured in light and in darkness at both sets of experimental conditions: 5 mM $NaHCO_3$ at pH 7.6 and 1 mM $NaHCO_3$ at pH 7.0. There were strong differences in the ^{35}S uptake between these two sets, but no significant differences between the values in light and darkness.

It seems evident from the data in Table I and Fig. 4 that the sulfite concentrations in chloroplasts were approximately proportional to their concentration in the medium, in any case about 25%. On the other hand, the ratio of ^{35}S concentration in the medium to the one in whole protoplasts increased with increasing sulfite concentration in the medium. With other words: the higher the sulfite concentration in the medium the higher the proportion of ^{35}S in the protoplasts which penetrated into the chloroplasts.

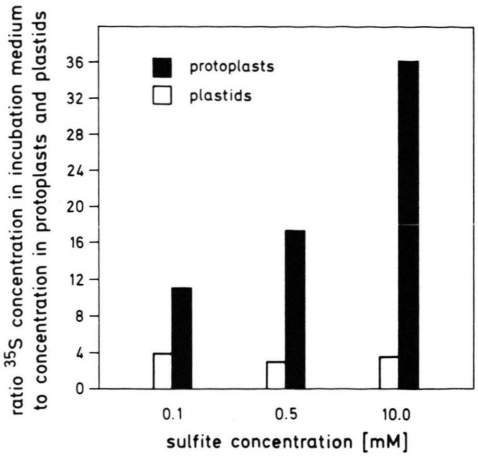

Fig. 4. Ratio of ^{35}S concentration in the incubation medium to the one in protoplasts and chloroplasts. The data in the diagram are mean values of the data in Table I.

Discussion

In experiments with isolated spinach chloroplasts it was demonstrated [14] that the uptake of sulfite proceeded linearly during the first 30 sec. After 30 sec the uptake dropped. During the next 10 min of exposure to sulfite no further significant increase in the uptake of $^{35}SO_3^{2-}$ was observed [15]. Very similar results were obtained in experiments with *Chlorella* [16]: The uptake was very rapid during the initial phase (1 min) and then almost no change occurred during following 4 min. In our experiments no significant differences in uptake of $^{35}SO_3^{2-}$ were observed after 5, 10 and 60 min of incubation. The conclusion is therefore that the uptake was very rapid at the beginning, then a balanced state was established between protoplasts and the medium. Similar results were reported also with the transportation of CO_2 into chloroplasts [17]: A kinetic of bicarbonate uptake could not be established because the penetration of the compound through the plastid envelope was too rapid.

Also the efflux of sulfite was rapid in the first minutes in our experiments (Fig. 2). The efflux curves

Table I. Sulfite concentrations (mM) in protoplasts and their chloroplasts of *Avena sativa* leaf mesophyll cells. Protoplasts were incubated for 1 h in darkness or in light in media containing 0.1, 0.5, and 10.0 mM sulfite and 5 mM $NaHCO_3$ at pH 7.6 or 1 mM $NaHCO_3$ at pH 7.0. The volume of 10^6 protoplasts is about 23 µl and the one of the chloroplasts from 10^6 protoplasts about 2.5 µl [8].

Sulfite concentration in medium [mM]	Protoplasts				Chloroplasts			
	Dark		Light		Dark		Light	
	pH 7.6	7.0	7.6	7.0	7.6	7.0	7.6	7.0
	$NaHCO_3$ 5 mM	1 mM	5 mM	1 mM	5 mM	1 mM	5 mM	1 mM
0.1	0.006	0.008	0.004	0.018	0.020	0.032	0.021	0.029
0.5	0.020	0.025	0.012	0.060	0.124	0.197	0.132	0.230
10.0	0.194	0.237	0.120	0.551	2.045	3.251	2.150	3.531

for protoplasts and chloroplasts are quite similar. The somewhat higher efflux from protoplasts could be due to a higher permeability to SO_3^{2-} of the plasmalemma and tonoplast and perhaps the mitochondrial membranes than the one of the chloroplast envelope.

It is to be noted that ^{35}S concentrations in protoplasts were much lower than in chloroplasts; this was the case for every sulfite concentration in the medium. The reason could be an active transport component in the case of uptake into the chloroplasts (*cf.* [14]) and/or a metabolic sink for ^{35}S inside the chloroplast, removing a considerable amount of $^{35}SO_3^{2-}$ from the diffusion equilibrium. The similar shape of efflux curves (Fig. 2) for chloroplasts and protoplasts suggests that at least the efflux of unfixed ^{35}S was quite similar, even not completely identical, through all cellular membranes, perhaps a simple diffusion. Also CO_2 transport through chloroplast envelope was considered to be a diffusion process [17].

This accumulation of sulfur compounds in plastids in comparison to other cell compartments is in accordance with results of other authors [10, 18].

As stated in an earlier paper [2], pH and bicarbonate concentration played an important role in the effect of sulfite on photosynthesis. The method of rapid protoplast fractionation allowed the conclusion that this influence of bicarbonate was not caused by differences in the sulfite uptake into protoplasts or chloroplasts: the ^{35}S uptake was only insignificantly lower at high (5 mM) than at low (1 mM) bicarbonate concentration in the medium.

The protective effect of higher CO_2 concentrations against SO_2 induced inhibition of photosynthesis should have other reasons, *e.g.* reducting of stomatal diffusion [19] (not in experiments with protoplasts) or the competitive exclusion of SO_2 in the RuBP-carboxylase reaction [20, 21] and activation [22].

The higher uptake of sulfite at lower pH values of the same medium can be explained by the fact that it is uncharged sulfite which is transported through membranes [15, 16, 23]. The same is true also for CO_2/bicarbonate [17, 24]. An increased inhibitory effect of SO_2 or sulfite on photosynthesis at lower pH values was reported by several authors [23, 25–27].

In our material and under our conditions there were no distinct differences between the uptake of ^{35}S in light and darkness (Table I). This is in contradiction to former reports [14, 28] that the mechanism of ^{35}S uptake was linked with the CO_2 uptake in the course of photosynthesis. The inconsistency may be due to the much lower light intensity in our experiments and/or to the different plant material.

Acknowledgements

Z. M. is indebted to Alexander von Humboldt-Stiftung for a grant; he was supported also by the Polish Governmental Project No. CPBP 05.02.03.12.

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