Uptake and Efflux of $[^{35}\text{S}]$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


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

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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 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.

**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% cellulose (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$_2$SO$_3$ 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 sulfate 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 x g, the residue was suspended in 20 ml of sulfate-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 mM Tris-HCl buffer (pH 7.6) containing 1 mM 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 x 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 organelles 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 organelles 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 14C-labelled sucrose, which was added immediately before fractionation. The actual amount of 35S 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 35S 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 [35S]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 35S efflux from protoplasts they were submitted to the washing procedure after 1 h of incubation. As can be seen in Fig. 2, 35S 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 35S content was about 25 to 30% of the original content. Throughout the experiments the 35S concentration in chloroplasts was 3 to 4 times higher than in whole protoplasts. The 35S efflux rate from protoplasts was insignificantly higher than from chloroplasts.

The effects of the pH value and NaHCO3 concentration in the medium on the uptake of 35S 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 NaHCO3 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 m M 35S sulfite and 1 mM NaHCO3 at pH 7.0.](image)

![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 NaHCO3, pH 7.0; b: media of pH 7.0 or 7.6. The data of b are mean values of measurements made at NaHCO3 concentrations of 1, 5 and 10 mM.](image)
5 mM at pH 7.0 had only a slight reducing effect on the levels of labelled sulfur in protoplasts and chloroplasts. At all NaHCO₃ concentrations the uptake of ³⁵S 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 NaHCO₃ at pH 7.6, as conditions inhibiting ³⁵S uptake, and 1 mM NaHCO₃ 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 ¹⁴C₀₂ uptake and the last strongly inhibiting this uptake [13]. For all three sulfite concentrations the ³⁵S uptake was measured in light and in darkness at both sets of experimental conditions: 5 mM NaHCO₃ at pH 7.6 and 1 mM NaHCO₃ at pH 7.0. There were strong differences in the ³⁵S 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 ³⁵S 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 ³⁵S in the protoplasts which penetrated into the chloroplasts.

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 ³⁵SO₃²⁻ 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 ³⁵SO₃²⁻ 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 CO₂ 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>
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<tr>
<th>Sulfite concentration in medium [mM]</th>
<th>Protoplasts</th>
<th>Chloroplasts</th>
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<tr>
<td></td>
<td>Dark pH 7.6</td>
<td>Light pH 7.0</td>
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<tr>
<td>0.1</td>
<td>0.006</td>
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<td>0.5</td>
<td>0.020</td>
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<td>10.0</td>
<td>0.194</td>
<td>0.120</td>
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for protoplasts and chloroplasts are quite similar. The somewhat higher efflux from protoplasts could be due to a higher permeability to $\text{SO}_3^{2-}$ of the plasmalemma and tonoplast and perhaps the mitochondrial membranes than the one of the chloroplast envelope.

It is to be noted that $^{35}\text{S}$ 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 $^{35}\text{S}$ inside the chloroplast, removing a considerable amount of $^{35}\text{SO}_3^{2-}$ from the diffusion equilibrium. The similar shape of efflux curves (Fig. 2) for chloroplasts and protoplasts suggests that at least the efflux of unfixed $^{35}\text{S}$ was quite similar, even not completely identical, through all cellular membranes, perhaps a simple diffusion. Also $\text{CO}_2$ 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 $^{35}\text{S}$ uptake was only insignificantly lower at high (5 mM) than at low (1 mM) bicarbonate concentration in the medium.

The protective effect of higher $\text{CO}_2$ concentrations against $\text{SO}_2$ 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 $\text{SO}_2$ 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 $\text{CO}_2$/bicarbonate [17, 24]. An increased inhibitory effect of $\text{SO}_2$ 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 $^{35}\text{S}$ in light and darkness (Table I). This is in contradiction to former reports [14, 28] that the mechanism of $^{35}\text{S}$ uptake was linked with the $\text{CO}_2$ 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.

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