Studies of Sulfate Utilization by Algae

8. THE UBICITY OF SULFATE REDUCTION TO THIOSULFATE

ROBERT C. HODSON and JEROME A. SCHIFF
Department of Biology, Brandeis University, Waltham, Massachusetts 02154

ABSTRACT

Cell-free extracts from several microorganisms, when prepared by methods originally devised for Chlorella pyrenoidosa (Emerson strain 3) and incubated anaerobically with ATP, Mg++, and 2,3-dimercaptopropan-1-ol, are capable of reducing sulfate-S to thiosulfate. These microorganisms include, in addition to C. pyrenoidosa (Emerson strain 3), several other strains of C. pyrenoidosa, Chlorella protothecoides, Chlorella vulgaris, Anacystis sp., Chlamydomonas reinhardi, Escherichia coli, Salmonella typhimurium, and baker's yeast. Three of these organisms, E. coli, S. typhimurium, and baker's yeast, were previously reported by others to reduce sulfate to sulfite. Moreover, three mutant strains of S. typhimurium (Ba-25, Ce-563, and Be-482) previously reported by other workers to be unable to reduce sulfate to sulfite also cannot form thiosulfate, and one mutant strain (Cd-68) reportedly able to form sulfite can also form thiosulfate. Taken together, this suggests that thiosulfate-forming activity may be a common feature of sulfate-reducing systems, and it may be present in enzymatic systems previously thought to be forming sulfite. Reasonably conclusive identification of thiosulfate is provided by ion exchange chromatography and by paper electrophoresis; the ambiguities associated with other analytical methods are discussed.

Previous reports in this series (15, 21, 25) have described a cell-free system from Chlorella pyrenoidosa (Emerson strain 3) which is capable of reducing sulfate to thiosulfate, via adenosine 3'-phosphate 5'-phosphosulfate, when fortified with ATP, Mg++, and a reductant. Reduced pyridine nucleotides or a thiol such as 2,3-dimercaptopropan-1-ol can serve as the reductant. This system is routinely assayed by the formation of acid-volatile radioactivity, and the product, before acidification and in the absence of added carrier, has been identified as thiosulfate by ion exchange chromatography and paper electrophoresis (21).

Since C. pyrenoidosa (Emerson strain 3) is the only assimilatory sulfate reducer reported to yield extracts which form thiosulfate from sulfate, we wondered whether the activity is unique to this organism. Of special interest were those cell-free systems already reported to be capable of forming sulfite, or sulfide, such as yeast (9, 10, 30), Escherichia coli (6, 23), and Salmonella (4). In this paper we report that crude cell-free extracts from various algae, bacteria, and yeast can reduce sulfate-S to acid-volatile radioactivity under our conditions, and in every case where sufficient product for identification was obtained, the major source of acid-volatile radioactivity was identified as thiosulfate. A brief account of this work has already appeared (11).

MATERIALS AND METHODS

All materials and methods not specifically described here are to be found in other papers in this series (12, 15, 21, 25).

Cell-free extracts were prepared at 0 to 4 C and were used immediately after preparation. All buffers used in the preparation of extracts contained 1.5 mm BAL. Protein was determined turbidimetrically (12).

Preparation of Chlorella Extracts. Cultures were grown under aseptic conditions photoautotrophically with shaking (14, 25). Our usual method of preparing Chlorella extracts (12, method A) was employed, and the supernatant obtained after the high speed centrifugation step was diluted with 4 volumes of 200 mm tris-HCl, pH 9.0, before being used as enzyme in incubations.

Preparation of Chlamydomonas, Anacystis, and Euglena Extracts. The procedures described above for Chlorella were used with the following changes.

Chlamydomonas reinhardi Danceard strain Y1 Sagar was grown in the medium of Levine (20) with the source of iron and trace elements the same as for the Chlorella medium (25), and supplemented with 0.4% (w/v) yeast extract (Difco).

Anacystis sp. strain 6311 was grown in the medium of Hughes et al. (16) with the following changes (method of M. M. Allen, R. Y. Stanier; R. Y. Stanier, personal communication). NaN03 (1.5 g/liter), ferric citrate (0.06 g/liter), and citric acid (0.06 g/liter) were increased to the parenthetical values, and 1 ml/liter of a trace element solution containing, per liter, H2BO3 (2.86 g), MnCl2·4H2O (1.81 g), ZnSO4·7H2O (0.222 g), MoO3 (85%) (0.0177 g), CuSO4·5H2O (0.079 g), and Co(NO3)2·6H2O (0.0494 g) was employed.

Euglena gracilis Klebs var. bacillaris Pringsheim was grown in medium B of Hutner as reported by Greenblatt and Schiff (8) with 5-fold more FeCl3·6H2O, 1.36 g of KH2PO4, 1.74 g of K2HPO4, and with the pH adjusted to 6.8 with NaOH before autoclaving (J. Diamond, personal communication). The cells were broken at 3 tons of pressure.

Preparation of Salmonella Extracts. Salmonella typhimurium C527 (derived from strain LT-2 and requiring histidine) was

Received for publication June 8, 1970

1 Supported by Grant GB 4231 from the National Science Foundation.
2 Postdoctoral Trainee, United States Public Health Service. Present address: Department of Biological Sciences, University of Delaware, Newark, Del. 19711.

2 Abbreviations: BAL; 2,3-dimercaptopropan-1-ol; PAPS: adenosine 3'-phosphate 5'-phosphosulfate.
grown with shaking at 37°C in medium E of Vogel and Bonner (29) supplemented with 20 μg of histidine per ml. Log phase cells were harvested by centrifugation at 6,000g for 15 min. The cells were washed once with 50 mM tris-HCl, pH 8.0, and the pellet was suspended in about 5 volumes of the same buffer. After breaking the cells with the French pressure cell at 9 tons, the extract was centrifuged at 18,000g for 15 min, and the supernatant solution was centrifuged at 86,000g for 1 hr. The supernatant solution constituted the enzyme extract.

Cysteine mutants of S. typhimurium, Ba-25, Cd-68, Ce-363, and Bc-482, were obtained from P. E. Hartman. The expected nutritional responses (22) for these mutants were confirmed by

Table I. Reduction of $^{35}$SO$_4^{2-}$ to Acid-volatile Radioactivity in Crude Extracts from Various Microorganisms

<table>
<thead>
<tr>
<th>Group</th>
<th>Organism</th>
<th>Source</th>
<th>Acid-volatile Radioactivity</th>
<th>$^{35}$S $\times 10^{2}$ cpm/mg protein/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Chlorella pyrenoidosa</td>
<td>Strain 3 (Emerson)</td>
<td>ATCC</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strain 7516</td>
<td>ICCC</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strain 343</td>
<td>ICCC</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strain 252</td>
<td>ICCC</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strain 11469</td>
<td>ATCC</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chlorella protothecoides strain 25</td>
<td>J. F. Thompson</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chlorella vulgaris var. viridis</td>
<td>ATCC</td>
<td>61</td>
</tr>
<tr>
<td>II</td>
<td>Anacystis sp. strain 6311</td>
<td>M. M. Allen and R. Y. Stanier</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Euglena gracilis var. bacillaris</td>
<td>R. Y. Stanier</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chlamydomonas reinhardtii strain Y1</td>
<td>R. P. Levine</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Escherichia coli strain A232</td>
<td>D. H. Gillespie</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Salmonella typhimurium</td>
<td>Strain C527 (&quot;wild-type&quot;)</td>
<td>D. Botstein</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strain Ba-25</td>
<td>P. E. Hartman</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strain Cd-68</td>
<td>P. E. Hartman</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strain Ce-363</td>
<td>P. E. Hartman</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strain Bc-482</td>
<td>P. E. Hartman</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Baker’s yeast</td>
<td>...</td>
<td>6.3</td>
</tr>
</tbody>
</table>

1 ATCC: American Type Culture Collection; IUCC: Indiana University Culture Collection.

plating. The same techniques were used as for strain C527 except that the growth medium was supplemented with 40 μg of L-djenkolic acid per ml and histidine was omitted.

Preparation of Escherichia coli Extracts. E. coli A232 (3) was grown with shaking at 37°C in 0.8% (w/v) nutrient broth. Log phase cultures were harvested and washed in the same way as for Salmonella. The cells were suspended in 3 volumes of the pH 8 buffer and broken by a lysozyme freeze-thaw procedure (17) as follows. The cell suspension was made 1 mg/ml in lysozyme and incubated for 5 min at 30°C. The cells were lysed by a rapid freeze-thaw cycle, and the extract was centrifuged at 28,000g for 15 min. The supernatant solution constituted the enzyme extract.

Preparation of Yeast Extracts. Fourteen grams of dried baker’s yeast (Fleischmann’s active) were suspended in about 100 ml of 50 mM tris-HCl, pH 8.0. Portions of this suspension (about 30 ml) were shaken with 50 g of acid-washed and neutralized glass beads (0.1 mm diameter) for 1 min in a chilled Braun cell homogenizer (model MSK, Will Scientific Co.). The extract

Fig. 1. Electrophoretic separation of cell-free extracts from several microorganisms incubated with $^{35}$SO$_4^{2-}$. The incubation mixtures were the same as those described in Table I. Electrophoresis conditions were: pH 5.8, 5°C, 1800 v (40 v/cm), and 40 min.
was centrifuged at 10,000 g for 10 min, and then at 86,000 g for 1 hr. The supernatant solution constituted the enzyme extract.

RESULTS

Extracts from the various algae, bacteria, and yeast were first tested for their ability to reduce $^{35}$SO$_{4}^{2-}$ to acid-volatile radioactivity (Table I). All extracts from wild-type strains (wild-type with respect to sulfate reduction) were active, although extracts from *Euglena* were always much less active than the others. Of the four strains of *Salmonella* impaired in sulfate reduction (4) which were tested, only extracts from strain Cd-68 were active.

Although with extracts from *Chlorella pyrenoidosa* (Emerson strain 3) thiosulfate is the major acid-volatile product of sulfate reduction (21), this could not be assumed to be true with extracts from other organisms. Indeed, several oxidation states of inorganic sulfur yield volatile products when treated with acid, including sulfite, thiosulfate, and sulfide. Furthermore, the addition of carrier thiosulfate, sulfite, or sulfide in the usual diffusion assay invites chemical exchange with the true product (1, 28). We sought, therefore, to identify the product which yields acid-volatile radioactivity by our usual methods of ion exchange column chromatography and paper electrophoresis in the absence of added carrier (21).

Paper electrophoresis of all *Chlorella* incubation mixtures (Fig. 1) revealed that the major radioactive species present were PAPS, sulfite, and thiosulfate. (This confirms the usual composition of *Chlorella* incubation mixtures already reported using ion exchange column chromatography [21].) Of these radioactive compounds, only thiosulfate is capable of yielding acid-volatile radioactivity. Similarly, incubation mixtures of extracts from *Chlamydomonas*, *Anacystis*, *Salmonella* (C527 and Cd-68), *Escherichia* and baker’s yeast, examined by ion exchange chromatography (Fig. 2) and paper electrophoresis (Fig. 1), revealed that thiosulfate was the major acid-volatile species present. Sulfite and sulfide were definitely absent, and there was no appreciable radioactivity at the origin on the electrophoresis papers which would be expected for a protein-bound form of radiosulfur (27). *Euglena* extracts were not active enough to permit identification of the product(s).

**DISCUSSION AND CONCLUSIONS**

The data we have presented in this paper show that thiosulfate is the major acid-volatile product formed when extracts from various species and strains of *Chlorella*, as well as from *Euglena*, *Chlamydomonas*, *Anacystis*, *Escherichia*, *Salmonella*, and baker’s yeast, are prepared and incubated under the conditions we originally devised for *Chlorella pyrenoidosa* (Emerson strain 3). Sulfite and sulfide are not found. Our methods do not involve the inclusion of carrier sulfite, sulfide, or thiosulfate in the incubations or subsequent separations, in order to avoid exchange with the compound(s) actually formed from PAPS. Furthermore, acidification is not involved in this identification, which eliminates confusion in identifying the source of SO$_{2}$ (which might be from either sulfite or thiosulfate) and also prevents the acid-catalyzed reaction between sulfite and sulfide known to form a mixture of products, including sulfur, resembling Wackenroder’s liquid (28).

Previous work under a variety of extraction and incubation conditions has led to suggestions that sulfite, sulfide, or both are formed in extracts from some of the organisms mentioned above, particularly *Escherichia coli* (6, 23) and *Salmonella* (4), as well as several other organisms summarized in the review of Peck (24). Hilz and co-workers (9, 10) and Bandurski and co-workers in their early papers (30) suggested that extracts from baker’s yeast form sulfite. In a later paper Torii and Bandurski (27) modified this in favor of a bound form of sulfur which could yield sulfite under certain conditions. Extracts of higher plants have also been examined for sulfate reduction, and the conclusion has been drawn that sulfite is the major product (2, 26).

Most of these identifications of sulfite or sulfide as the major acid-volatile product formed from sulfate have depended upon the addition of carrier prior to analytical determinations. It is known, however, that sulfite exchanges readily with the SO$_{3}$ portion of thiosulfate (1, 29), and thiosulfate itself yields SO$_{2}$ upon acidification. The identification of sulfite is rendered somewhat ambiguous by its reaction with sulfite (28), on acidification, and by its ready exchange with the SH sulfur of thiosulfate (1). It would seem that some of these identifications are not unambiguous and it would be desirable to have separations based on ion exchange chromatography or electrophoresis in the absence of carrier.

In work on extracts of *E. coli* (6) and *Salmonella* (4), net amounts of sulfite have been shown to be formed from nonradioactive sulfate using the Grant procedure (7). This procedure involves deproteinization with mercuric chloride followed by determination of sulfite with fuchsine reagent. Kelly and Syret (18) have used mercuric chloride to selectively degrade thiosul
fate to sulfate and mercuric sulfide. Thus net sulfite formation, determined by the Grant procedure, may represent authentic sulfite formation, but the procedure would preclude the identification of any thiosulfate which might be formed.

Dreyfuss and Monty (4) have suggested that Salmonella mutants Ba-25, Ce-363, and Bc-482 are blocked for sulfate reduction by being unable to form sulfite, determined by the Grant procedure already mentioned, which they suppose to be an intermediate. Mutant Cd-68, although unable to grow on sulfate, was reported by these workers to form sulfite. It is interesting to reinterpret their conclusions based on our findings. We find that mutants Ba-25, Ce-363, and Bc-482 are unable to form thiosulfate while mutant Cd-68 can. It would appear that we are dealing with the same enzyme system, and, in view of our findings that thiosulfate rather than sulfate is formed, we wonder whether the Grant procedure was actually determining sulfite. It is also possible, of course, that a common intermediate formed from PAPS can donate its sulfur to the formation of either sulfite or thiosulfate depending on conditions, a suggestion made by Bandurski and by ourselves.

At present, we must conclude that systems forming thiosulfate certainly exist in the organisms reported in this paper, but whether other systems forming sulfite or sulfate are also present must await unambiguous identification of the products leading to acid-volatile radioactivity. Although we have been concerned only with assimilatory sulfate reducers, it is of interest that extracts from a dissimilatory sulfate reducer, Desulfovibrio vulgaris, are reported to form thiosulfite from sulfate (5, 19).

Acknowledgments—We are indebted to the following individuals for providing organisms for this work: M. M. Allen and R. Y. Stanier, Department of Microbiology, University of California; Richard C. Starr, Department of Botany, University of Indiana (Director of the culture collection); John F. Thompson, United States Plant, Soil and Nutrition Laboratory, Ithaca, New York; R. P. Levine, Harvard University; D. H. Gillespie, Department of Biology, Brandeis University; P. E. Hartman, Johns Hopkins University; and D. Botstein, Department of Microbiology, Massachusetts Institute of Technology. The expert technical assistance of Miss Jeannette Lemieux is gratefully acknowledged.

LITERATURE CITED