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Studies of Sulfate Utilization by Algae

10. NUTRITIONAL AND ENZYMATIC CHARACTERIZATION OF *CHLORELLA* MUTANTS IMPAIRED FOR SULFATE UTILIZATION[1]

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ABSTRACT

Seven mutants of *Chlorella pyrenoidosa* (Emerson strain 3) impaired for sulfate utilization have been isolated after treatment of the wild-type organism with nitrosoguanidine by replica plating on media containing thiosulfate and L-methionine. These mutants fall into three classes based on their ability to grow on sulfate, accumulate compounds labeled from sulfate-^{35}S, and reduce adenosine 3′-phosphate 5′-phosphosulfate-^{35}S (PAPS-^{35}S) to thiosulfate-^{35}S. Mutant Sat_2^- cannot grow on sulfate, but it accumulates thiosulfate-^{35}S and homocysteic acid-^{35}S from sulfate-^{35}S *in vivo*. In addition, extracts of mutant Sat_2^- reduce PAPS-^{35}S to thiosulfate-^{35}S, indicating the possession of enzyme fractions S and A, both of which are required for thiosulfate formation. Mutants Sat_1^-, Sat_3^-, Sat_4^-, Sat_5^-, and Sat_6^- cannot grow on sulfate, and their extracts lack the ability to reduce PAPS-^{35}S to thiosulfate-^{35}S. Mutant $Sat_7^-R_1$, a probable revertant, can grow on sulfate but still lacks the ability to reduce PAPS-^{35}S to thiosulfate-^{35}S *in vitro*. Complementation experiments *in vitro* show that the block in formation of acid-volatile radioactivity in every case is due to the absence of activity associated with fraction S. All mutants can grow on thiosulfate and all possess the activating enzymes which convert sulfate to PAPS. Through a comparison of nutritional and enzymatic characteristics, the first outlines of a branched and complicated pathway for sulfate reduction in *Chlorella* are beginning to emerge.

We have previously shown that a cell-free system from *Chlorella* will reduce sulfate via PAPS[3] to thiosulfate (17, 22, 30) which yields acid-volatile radioactivity. The conversion of PAPS to acid-volatile radioactivity requires two enzyme fractions, S and A (14). Although both sulfur atoms of thiosulfate can be utilized efficiently as sulfur sources for growth by *Chlorella* (16), and small amounts of thiosulfate can be detected in the free pools of cells grown on radioactive sulfate (22), the position of thiosulfate in the reduction pathway is still undetermined. With this problem in mind, we set out to isolate mutants of *Chlorella* which are blocked for sulfate utilization, since these might be expected to accumulate intermediates in reduction and to lack certain enzymatic steps, as has been found with similar mutants in other species (7, 18, 23, 33). In this paper we report the isolation and characterization of seven mutants of *Chlorella*, one of which accumulates thiosulfate. Several of the mutants lack enzyme fraction S, and through a comparison of nutritional and enzymatic characteristics the first outlines of a branched and complicated pathway are beginning to emerge. A preliminary account of this work has already appeared (12).

MATERIALS AND METHODS

All pertinent materials and methods not described here can be found in one of the accompanying papers (13, 14) or previous reports in this series (11, 16, 17, 22, 30). These include: the growth of *Chlorella* in photoautotrophic shake cultures (16, 30); preparation of crude extracts on a small scale (14); preparation of components S and A from wild-type (14); methods for feeding radioactive substrates to cell suspensions (16, 28); determination of protein in whole cells (16) and cell-free extracts (14); determination of radioactivity incorporated into the alcohol-insoluble fraction and into methionine and cystine of protein (16); incubation of enzyme extracts with $^{35}SO_4^{2-}$ and PAPS-^{35}S (14, 17); determination of PAPS-^{35}S (14, 17) and acid-volatile radioactivity (17) formed in enzyme incubations; the analysis of cell-free incubation mixtures by Dowex 1-NO_3^- chromatography (22) and paper electrophoresis (13, 22); and the preparation of PAPS-^{35}S with activating enzymes from *Chlorella* (11.)

The experimental organism was *Chlorella pyrenoidosa* Chick (Emerson strain 3).

Media. The composition of the basal (B) medium is given in Table I. Other media are as follows: BMT medium is B medium containing 2 mM sodium thiosulfate and 2 mM L-methionine sterilized separately by filtration; BG medium is B medium containing 0.1% (w/v) glucose and 2% (w/v) agar, both added before autoclaving; BGMT medium is BMT with 0.1% glucose and 2% agar added before autoclaving; and sulfur-free or SF medium is B medium with sodium sulfate omitted.

Induction and Selection of Mutants. Wild-type *Chlorella* was grown with shaking in 50 ml of B medium with 2% CO_2 in air and 500 ft-c of white fluorescent illumination. The cultures were used when they reached the stationary phase of growth, at which time there were 5 to 10 $\times$ 10^8 cells per ml. All organisms appeared to be at the single cell stage, and, judging from the absence of a shoulder in an untraviolet killing curve, they probably were also haploid.

Chlorella was treated with nitrosoguanidine, with the use of aseptic procedures essentially by Adelberg's method (1). One-milliliter aliquots of stationary phase culture were placed into each of several 125-ml Erlenmeyer flasks with 9 ml of B medium containing 1 mg of nitrosoguanidine (freshly prepared and

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[3] Abbreviation: PAPS: adenosine 3′-phosphate 5′-phosphosulfate.

sterilized by filtration). The flasks were placed in the dark and incubated at 25 C with gentle shaking. After 30 min, 1 ml was withdrawn from each flask and filtered on a Millipore membrane. The cells retained on the filter were washed with about 10 ml of B medium, and each disk (five in all) was placed in a separate 250-ml Erlenmeyer flask containing 20 ml of BMT medium. The cultures were grown photoautotrophically to the stationary phase in order to achieve a uniform population of cells and to allow expression of any mutations.

Aliquots from each of three cultures were suitably diluted in B medium, and about 300 cells (0.2 ml) were spread on each of 150 plates containing BGMT medium. These plates were incubated at 25 C under dim red fluorescent light (4 ft-c, Weston sunlight illumination meter) for 2 days and then under red light (83 ft-c) until the colonies reached 1 mm or so in diameter. Red light was used instead of white because we found that *Chlorella* cells grown on solid media containing methionine were killed by as little as 20 ft-c of white light whereas they were relatively insensitive to red light. This problem was not encountered with liquid cultures. The fact that a methionine product seems to photosensitize the cells may be related to the previous findings for *Chlorella pyrenoidosa* (28, 29) and *Euglena gracilis* (9) that methionine never accumulates in the free pools. These cells have probably evolved control mechanisms to ensure that significant accumulations of methionine do not occur in the free pools, and, therefore, photosensitizing substances are not produced in quantity.

The colonies were replicated onto plates containing BG and BGMT medium by means of two layers of sterile Whatman No. 1 filter paper mounted on round wooden pedestal blocks, and the replica plates were incubated under red light for 5 days. Those colonies growing well on BGMT but only slowly or not at all on the BG medium were picked and stabbed into BGMT plates. When sufficient inoculum was obtained, cells were transferred by means of a sterile loop to a drop of sterile water placed on a plastic Petri dish. These suspensions were then spotted onto BGMT and BG plates (first spot test). All isolates which grew equally well on BGMT and BG were discarded at this point. The remaining isolates were streaked onto BGMT, and three well isolated colonies were picked from each. Each of these colonies was inoculated into 2 ml of BMT medium contained in 13- × 100-mm culture tubes and incubated under red light. When sufficient growth appeared, each isolate was retested on plates of BGMT and BG medium (second spot test). Revertants were discarded and two isolates from each mutant strain were transferred to BGMT slants, grown for a few days, and then placed at 4 C for storage. These slants constitute our stock cultures, and they are retested and cultured about once a year.

The mutants are designated essentially by the system suggested for *Euglena* (31). Sat$^-$ indicates that the mutants are phenotypically unable to utilize sulfate as the sole source of sulfur for growth, and separate isolates are numbered serially. E indicates that they arose from the Emerson strain of *Chlorella pyrenoidosa*, Ng that the mutagen was nitrosoguanidine. Thus the first six mutants are designated Sat$_1^-$ENg through Sat$_6^-$ENg. Mutant 7 reverted phenotypically to wild type (although further characterization indicates that it is not identical with wild type, see below) and receives the designation Sat$_7^-$ENgR$_1$ to indicate that it is the first revertant from Sat$_7^-$, the original strain having been lost. These mutants will be referred to subsequently as Sat$_1^-$ through Sat$_6^-$ and Sat$_7^-$R$_1$.

Nutritional Tests. Two methods were used to test the nutritional requirements of the mutant strains. (*a*) Cells were transferred from slants to BMT shake cultures and grown to the stationary phase. These suspensions were diluted 10-fold with SF medium and spotted onto BG plates supplemented with the desired sulfur compound (sterilized by filtration). The plates were incubated under red light and observed after 5 and 14 days. (*b*) BMT shake cultures were harvested by centrifugation, and the cells were washed once with SF medium. Washed cells were suspended in fresh sterile SF medium at one-half the original cell density and incubated with shaking another 2 days. Some of these starved cells were inoculated into SF medium with the appropriate sulfur source in 250-ml Erlenmeyer flasks bearing a side arm, and growth was measured as apparent absorbance with a Klett-Summerson colorimeter fitted with a green (560 nm) filter (16).

Table I. *Composition of Basal (B) Chlorella Medium*

Chemical	Concn	Volume Added
	mM	ml/liter
KH_2PO_4	2.5	...
$Mg(NO_3)_2 \cdot 6H_2O$	2.0	...
KNO_3	5.0	...
K_2HPO_4	2.5	...
Na_2SO_4	2.0	...
$Ca(NO_3)_2$	0.25	...
Fe-Versenol[1]	...	1.0
Trace elements[2]	...	1.0

[1] To make Fe-Versenol, 59 g of Versenol 120 are dissolved in 500 ml of water, 17.7 g of $FeCl_2 \cdot 4H_2O$ are added, and the volume is made to 1 liter. The solution is aerated overnight and filtered, and the filtrate is used.

[2] One liter of trace element solution contains: H_3BO_3 (1.43 g), $Mn(NO_3)_2 \cdot 6H_2O$ (1.78 g), $ZnCl_2$ (50 mg), $Cu(NO_3)_2 \cdot 3H_2O$ (38 mg), and $H_2MoO_4 \cdot H_2O$ (10 mg).

Table II. *Nutritional Responses of the Mutants to Various Potential Sulfur Sources Provided on Plates*

Growth responses were rated on a scale from 0 to +++. All sulfur compounds were provided at a concentration of 2 mM, and all media contained sulfate.

Strain	Nutritional Response to Sulfur Source							
	SO_4^{2-}		Methionine		$S_2O_3^{2-}$		Methionine + $S_2O_3^{2-}$	
Days of growth	5	14	5	14	5	14	5	14
Sat$^-$ 1	0	0	0	0	+++	+++	+++	+++
2	0	0	0	+	+++	+++	+++	+++
3	0	+	0	++	+++	+++	+++	+++
4	0	0	0	0	+++	+++	+++	+++
5	0	0	0	++	+++	+++	+++	+++
6	0	0	0	+	+++	+++	+++	+++
7	++	+++	+++	+++	+++	+++	+++	+++
Wild type	+++	+++	+++	+++	+++	+++	+++	+++

Tracer Experiments *in Vivo*. All strains were grown to the late log phase in BMT shake cultures. Sulfur-starved cells were prepared as described above. Starved cultures were harvested by centrifugation and suspended in one-eighth the original volume of SF medium, and 5-ml portions were placed in 50-ml Erlenmeyer flasks and equilibrated with shaking for 1 hr at 26 C under ordinary laboratory lighting conditions.

Incorporation of radiosulfur into constituents of the soluble pools was determined essentially as described previously (16). After incubating cell suspensions with $^{35}SO_4^{2-}$ followed by an additional incubation with iodoacetamide (10 mM), the cells in a 0.5-ml sample of suspension were washed and subsequently freeze-thawed in 0.2 ml of water. Chromatography of a 25-μl aliquot of extract was carried out on Whatman No. 3MM filter paper,

Table III. *Distribution of Radioactivity from Sulfate-^{35}S in Sulfur-starved Mutant and Wild-type strains of Chlorella*

A 5-ml suspension of cells of each strain, containing about 4 mg of protein per ml, received 69.4 × 10^6 cpm and 0.5 μmole of $^{35}SO_4^{2-}$ for 3 hr.

Strain	$^{35}SO_4^{2-}$ Taken up	Alcohol-insoluble Fraction		Free Amino Compounds (Cysteine + GSH + homocysteine)
		Cystine + methionine	SO_4^{2-}	
Sat⁻	10^6 × cpm/mg protein	% of uptake		% of uptake
1	3.6	0.15	5.6	0.05
2	4.1	1.2	8.1	0.16
3	3.5	1.7	9.4	0.33
4	3.4	0.022	10	0
5	3.0	0.028	8.3	0
6	3.4	0.024	8.7	0
7	3.2	13	6.4	3.1
Wild type	3.3	35	8.1	19

developed in the first direction with methanol–*n*-butanol–benzene–water (2:1:1:1) and in the second direction with *n*-butanol–glacial acetic acid–water (5:1:2) (28). Radioactive areas were located with Kodak no-screen x-ray film (28), eluted, and counted by liquid scintillation spectrometry (16).

Uptake of $^{35}SO_4^{2-}$ was determined either from the amount of radioactivity removed from the medium, or by the total radioactivity accumulated in the cells. For the latter determination, portions of suspension (usually 0.1 ml) were diluted to 10 ml with ice-cold SF medium containing 10 mM sodium sulfate. Aliquots of the dilution (0.1 ml) were filtered on 25-mm diameter Millipore membranes and washed with 10 ml of the same medium. The filters were then glued to plastic planchets, dried gently under an infrared lamp, and counted with a Nuclear-Chicago gas flow detector (D-47) operating in the proportional range. Self-absorption was found to be negligible with the amounts of cell material used. Either method was appropriate since none of the strains excreted a significant amount of radioactively labeled products.

Cocrystallization of Homocysteic Acid with Radioactive Unknown. Homocysteic acid and radioactive unknown were dissolved in a minimal volume of water with boiling, and the hot solution was made 80% (v/v) with respect to ethanol. After this solution was made 0.1 N with respect to HCl, crystals appeared upon cooling to room temperature. Crystallization was completed overnight at −20 C. The material was collected by filtration on a Millipore membrane, washed with 80% ethanol, dried for 12 hr over solid NaOH *in vacuo*, and weighed.

RESULTS AND DISCUSSION

Nutritional Responses of Sat⁻ Mutants. Sulfur compounds other than sulfate which the Emerson strain of *Chlorella pyrenoidosa* is known to utilize for growth are limited in number and include only thiosulfate, cysteine *S*-sulfonate, cystine (and cysteine), methionine, and djenkolic acid (16). Thiosulfate and methionine were chosen for the selection of mutants unable to reduce sulfate because they provide sulfur at partially and fully reduced levels. From about 45,000 cells plated, 27 colonies were picked off the replica plates as possible mutants. Of these, 9 were selected from the first spot test as sulfateless (Sat⁻) mutants with a nutritional requirement for sulfur not satisfied by inorganic sulfate. Upon a second spot test, one of the isolates had reverted to wild type and was discarded. The remaining 8 isolates formed the initial collection of mutant strains.

Sometime during the storage and culture of the mutants, Sat_7^- and Sat_8^- acquired the ability to grow on sulfate. On the basis of various nutritional and enzymatic criteria, Sat_8^- apparently reverted entirely to wild type whereas Sat_7^- did not. Attempts to reisolate the original sulfateless mutant from these two revertants were unsucessful. Sat_8^- is not considered to be interesting and will not be discussed further. Among the other mutant strains, only Sat_3^- gave rise to occasional variants which were indistinguishable from $Sat_7^-R_1$ in nutritional and enzymatic tests. A reasonable explanation of this reversion pattern is that the original isolates of Sat_1^-, Sat_3^-, Sat_4^-, Sat_5^-, Sat_6^-, and Sat_7^- are actually double mutants (nitrosoguanidine is reported to give double mutants with reasonable frequency [1]). Soon after isolation, one of the mutant loci in Sat_7^- reverted to wild type leaving us with a single mutation in the recovered strain, $Sat_7^-R_1$. This would also explain why mutants similar to $Sat_7^-R_1$ occasionally arise in Sat_3^- and, perhaps, in other mutants.

Two different patterns of growth response on solid media may be discerned in the data of Table II. Sat_1^- through Sat_6^- do not grow at all on sulfate, and compared to wild type they grow only slowly on methionine. $Sat_7^-R_1$ can utilize sulfate and grows well on methionine. All strains are completely satisfied with media containing thiosulfate. Similar nutritional responses are obtained from these mutants in liquid shake cultures, although $Sat_7^-R_1$ and revertants of Sat_3^- show an initial growth lag on sulfate.

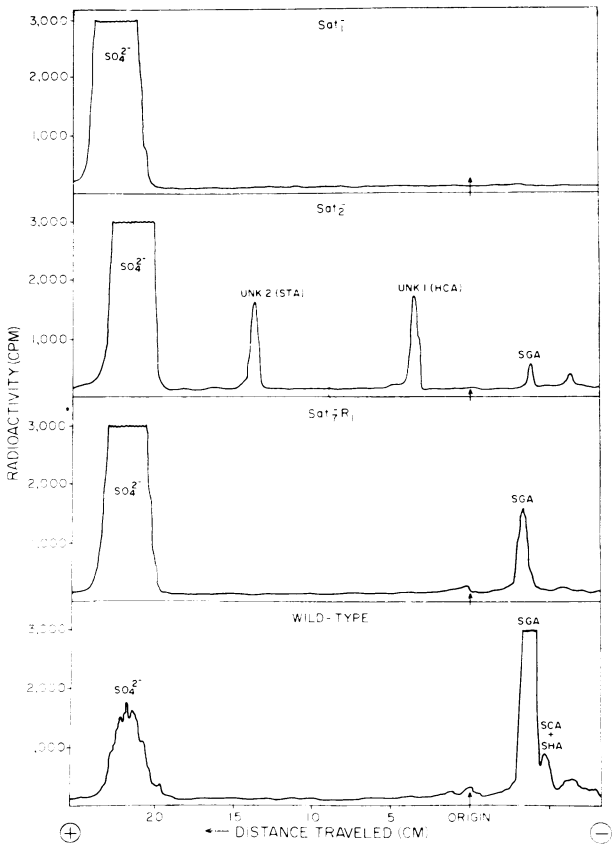

FIG. 1. Electrophoretic separation of the soluble pool constituents extracted from some mutant strains and from wild-type sulfur-starved *Chlorella* given sulfate-^{35}S. Extracts from the incubations presented in Table II (12 μl each) were subjected to electrophoresis at pH 2.0, 1800 v (45 v/cm) and 5 C for 35 min. Paper strips were scanned at a full scale setting of 3000 cpm, a 1-mm slit, and rate of 1.5 inches/min. STA: *S*-thiosulfate acetamide; HCA: homocysteic acid; SGA: *S*-glutathione acetamide; SCA: *S*-cysteine acetamide; SHA: *S*-homocysteine acetamide.

Uptake and Metabolism of $^{35}SO_4^{2-}$. The uptake of $^{35}SO_4^{2-}$ by suspensions of sulfur-starved cells and incorporation of radioactivity into constituents of the soluble pools and alcohol-insoluble fraction are presented in Table III and Figure 1. All of the mutant strains can take up sulfate (Table III). This is not unexpected, since there is some evidence for a common mechanism for the transport of thiosulfate and sulfate into *Chlorella* (Hodson and Schiff, unpublished data) and into *Salmonella* (6). Sat_1^- through Sat_6^-, however, are drastically impaired in their ability to incorporate radioactivity from $^{35}SO_4^{2-}$ into cysteine, homocysteine, and glutathionine (separated as their iodoacetamide derivatives) in the soluble pools (Table III and Fig. 1), and into cystine and methionine (separated as cysteic acid and methionine sulfone) of the protein fraction (Table III). In contrast to this, they are not impaired in their ability to incorporate radioactivity into certain alcohol-insoluble substances, presumably sulfate esters of carbohydrates, which yield $^{35}SO_4^{2-}$ upon acid hydrolysis (Table III). $Sat_7^-R_1$ is notably different from the other mutant strains and is similar to wild type in its ability to reduce sulfate. On the basis of the above labeling patterns and over-all nutritional requirements (Table II), the mutant strains can be divided into 2 distinctly separate groups. Group 1 is comprised of Sat_1^- through Sat_6^-, and group 2 contains $Sat_7^-R_1$.

Sat_2^-, unlike any of the other strains, accumulates two acidic radioactive substances in the soluble pools which are not found in wild type (Fig. 1). Enough labeled material has been obtained from sulfur-starved cells fed sulfate-^{35}S to allow a firm identification of both unknowns.

Identification of Unknown 1 of Sat_2^- as Homocysteic Acid. If iodoacetamide was omitted, a radioactive compound with the same electrophoretic properties as unknown 1 was present in extracts of Sat_2^-. This indicated that unknown 1 probably did not contain a sulfhydryl group. This unknown gave a color reaction with ninhydrin, and its electrophoretic mobility was dependent on pH in the range of 2 to 8.1, properties exhibited by amino acids. The anionic nature of this unknown at pH 2 and the presence of sulfur in a chemical form other than sulfhydryl indicated that the unknown might contain an oxide of sulfur, such as a sulfate ester ($R\text{-}O\text{-}SO_3^-$), thiosulfonate ($R\text{-}S\text{-}SO_3^-$), or sulfonate ($R\text{-}SO_3^-$). Complete stability of the unknown in boiling 0.1 N HCl ruled out a sulfate ester. The possibility of a thiosulfonate functional group was eliminated by the lack of reaction with performic acid (23). Amino acids containing a sulfonate group which suggested themselves were cysteic acid and homocysteic acid. The unknown cochromatographed with homocysteic acid in three different solvent systems, butanol–pyridine–water (1:1:1), methanol–butanol–benzene–water (1:1:1:1), and butanol–acetic acid–water (5:1:2), and showed coelectrophoresis with homocysteic acid at pH 2.0, 5.8, and 8.1. Final identification of unknown 1 as homocysteic acid (without specifying the D or L isomer) was obtained by cocrystallization of radioactively labeled material with authentic L-homocysteic acid to constant specific radioactivity (Table IV).

Table IV. *Cocrystallization of Unknown 1 and Authentic L-Homocysteic Acid*

Stage	Total Homocysteic Acid	Total Radioactivity	Specific Radioactivity
	mg	$10^{-6} \times$ *cpm*	*cpm/mg*
Starting mixture	500	4.78	9570
First crystallization	533	3.42	6410
Second crystallization	449	3.32	7390
Third crystallization	410	3.03	7380
Fourth crystallization	379	2.87	7560

Table V. *Enzymatic Activities in Crude Extracts of Mutant and Wild-type Chlorella*

Additions to the reaction mixture were: *Chlorella* extract (containing about 2 mg of protein per ml) (1.0 ml), $MgCl_2$ (50 μmoles), tris-base (500 μmoles), mercaptoethanol (60 μmoles), and either carrier-free $H_2{}^{35}SO_4$ (9.39×10^6 cpm) plus ATP (10 μmoles) or PAPS-^{35}S (0.9 μmole, 8.37×10^5 cpm) in a total volume of 2.35 ml. Incubation was for 1 hr at 30 C under N_2. The crude extracts were freshly prepared in 0.1 M tris-HCl, pH 7.0, containing 20 mM mercaptoethanol, and centrifuged at 86,000*g* for 1 hr before use. Thiosulfate was measured indirectly as acid-volatile radioactivity, and its identification was confirmed by paper electrophoresis at pH 5.8. PAPS-^{35}S was measured directly after electrophoresis (17).

Strain	Enzymatic Activity		
	$^{35}SO_4^{2-} \rightarrow {}^{35}S_2O_3^{2-}$	$^{35}SO_4^{2-} \rightarrow$ PAPS-^{35}S	PAPS-$^{35}S \rightarrow {}^{35}S_2O_3^{2-}$
	$10^{-3} \times$ *cpm per mg protein per hr*		*nmoles per mg protein per hr*
Sat⁻			
1	2.1	1424	1.5
2	1167	1557	103
3	5.9	1731	1.5
4	1.4	1914	1.7
5	1.7	1558	1.3
6	1.7	1645	1.3
7	1.1	1813	1.4
Wild type	1170	1673	76

Homocysteic acid was highly labeled when sulfate-^{35}S was supplied to cells of Sat_2^-. Under these conditions, the pools of reduced sulfur compounds showed very low radioactivity, consistent with the fact that Sat_2^- cannot grow on sulfate as the sole sulfur source. Taken together, this indicates that homocysteic acid is formed rather directly from sulfate without prior reduction to the thiol level and reoxidation. Formation of sulfonic acids from sulfate by *Chlorella* is already known from previous work on the formation of sulfolipids and other related compounds (3, 4). One possible mechanism for the formation of such compounds (other than the transfer of the SO_3 group from PAPS to phosphoenolpyruvate suggested by Davies *et al.* [5]) would be the reaction of PAPS with the appropriate *O*-succinyl or *O*-acetyl carbon skeleton, homoserine in the case of homocysteic acid and glucose in the case of the sulfolipid. The displacement of *O*-acetyl and *O*-succinyl groups by thiols or H_2S has been demonstrated (8, 19–21, 37), but a similar reaction with PAPS has not been attempted; presumably a reduction would be involved.

Identification of Unknown 2 of Sat_2^- as S-Thiosulfate Acetamide. The spot corresponding to unknown 2 was found only if the cells were treated with iodoacetamide. In addition to this evidence for a sulfhydryl group, a sulfonic acid group was implicated from the anionic character of this unknown at low pH. The electrophoretic mobility of unknown 2 was relatively unchanged in the pH range of 2 to 8.1, suggesting that this compound was not an amino acid. After oxidation with performic acid, almost all the radioactivity could be recovered as sulfate. These properties appeared to be similar to those expected of thiosulfate acetamide or a compound with a similar structure. Indeed, unknown 2 and *S*-thiosulfate acetamide showed coelectrophoresis at pH 2.0, 5.8, and 8.1 and cochromatographed in the same solvents employed in the identification of unknown 1. Thus unknown 2 represents the acetamide derivative of the thiosulfate accumulated by this mutant.

The accumulation of thiosulfate by this mutant provides ad-

Table VI. *Complementation of Components Converting PAPS-^{35}S to Acid-volatile Radioactivity*

The complete system contained: $MgCl_2$ (25 μmoles), tris-HCl, pH 9.0 (100 μmoles), PAPS-^{35}S (2057 cpm/nmole, 0.5 μmole), mercaptoethanol (100 μmoles), and, where indicated, crude mutant extract (containing about 6.0 mg of protein per ml) (0.2 ml), fraction S from wild type (containing about 3 mg of protein per ml) (0.2 ml), and fraction A from wild type (containing about 1.5 mg of protein per ml) (0.2 ml) in a total volume of 1.175 ml. Incubation was for 1 hr at 30 C under N_2. The mutant extracts were added as the 0 to 50% saturated ammonium sulfate precipitate from crude extracts.

Enzyme Fraction(s) Incubated	Activity					
	Sat_1^-	Sat_3^-	Sat_4^-	Sat_5^-	Sat_6^-	Sat_7^-
	nmoles PAPS-^{35}S to acid-volatile radioactivity					
Mutant extract	0.46	0.046	0.21	0.15	0.10	0.32
Mutant extract + S	69	64	71	72	51	76
Mutant extract (heated) + S	3.2	2.8	1.4	3.0	2.5	3.0
Mutant extract + A	25	24	25	30	19	25
S alone	3.9					
A alone	19					
S + A	86					

ditional evidence that thiosulfate may be a normally occurring intermediate in sulfur metabolism in this organism and suggests that the enzyme system forming it, already described for *Chlorella* extracts, may have physiological significance.

When thiosulfate is fed to intact cells, it appears to be immediately dismuted into moieties at the oxidation levels of sulfate and sulfide (16). Sat_2^- appears to be unable to dismute endogenous thiosulfate, since thiosulfate accumulates. Paradoxically, exogenous thiosulfate is readily used for growth by this mutant.

Enzymatic Activities of Mutants. Since crude, cell-free extracts of wild type are capable of reducing sulfate via PAPS to thiosulfate, it was of interest to examine extracts of the mutant strains for these enzymatic activities. The results are given in Table V. Only Sat_2^- yielded extracts capable of reducing sulfate to thiosulfate. Compared to wild-type extracts, those from Sat_2^- were equally active in this reduction. The missing enzymatic step in extracts of the other mutants is seen to be in the reduction of PAPS rather than in its formation. Even though $Sat_7^-R_1$ utilizes sulfate, the absence of thiosulfate formation in cell-free extracts indicates that it did not revert entirely to wild type.

Since all mutants are capable of forming PAPS (Table V), and since they also accumulate appreciable amounts of bound sulfate which can be released by acid hydrolysis (Table III), these mutants possess the enzymes for converting sulfate via adenosine 5′-phosphosulfate and PAPS to sulfate esters.

Since the enzymatic reduction of PAPS to acid-volatile radioactivity in extracts of wild type has at present been resolved into two separate and necessary protein fractions, called component S and component A (14), it was of interest to determine which of these activities was absent from extracts of mutant strains unable to reduce PAPS to thiosulfate. Crude extracts were prepared from each of these mutant strains. These extracts were fortified with S or A obtained from wild type as previously described (14) and incubated with PAPS-^{35}S. The results of such a complementation experiment *in vitro* are given in Table VI. Component S complements each of the mutant strains for the reduction of PAPS to thiosulfate, whereas component A does not. That the extract contributes an enzymatic component is seen from the failure of heated extracts to complement S. Thus, it appears that each of the mutant strains unable to reduce PAPS to thiosulfate *in vitro* contains A but lacks the active component(s) of S.

CONCLUSIONS

A Tentative Pathway of Sulfate Reduction in *Chlorella*. In Figure 2 we present a synthesis of our current information (15). This synthesis is based on the previous suggestions of Dreyfuss and Monty (7) and does not seem to be incompatible with their findings using *Salmonella*, or with those of Bandurski and coworkers for yeast (2, 34, 36) or the suggestions of Thompson (35). It also incorporates some earlier ideas of Ragland and Liverman (27).

The Main Line. The main line of sulfate reduction is thought to proceed through adenosine 5′-phosphosulfate and PAPS, the usual sulfate-activating steps. The sulfur moiety of PAPS is assumed to be transferred reductively to a carrier (—SO_3^-). This intermediate might be a protein-bound thiosulfonate of the type previously suggested by Hilz *et al.* (9) and Bandurski *et al.* (2, 36, 38) (P-S-SO_3^-). It appears that thiosulfate might be formed by elimination from a protein-bound thiosulfonate, particularly at high pH (32). This intermediate (—SO_3^-) would be further reduced to the thiol level either by formation of free sulfite and action of sulfite reductase or by reduction of the bound species to a bound form of sulfide which could serve as a precursor of the sulfur-containing amino acids (see Fig. 2). *Chlorella* mutants Sat_1^-, Sat_3^- to Sat_6^-, and Sat_2^- are thought to be blocked in the conversion of (—SO_3^-) to (—S^-) as shown.

The Side Paths. Sulfate esters are assumed to arise by sulfate transfer from PAPS as in other systems (26, 34) and to yield free sulfate on hydrolysis.

The oxidation of reduced sulfur compounds back to sulfate is shown since we have found that methionine-S^{35} is rapidly oxidized to sulfate by *Chlorella* cells (Diamond, Mather, Hodson, and Schiff, unpublished).

The bound intermediate of the main line (—SO_3^-) is thought also to be the source of the SO_3 moiety of thiosulfate, the S moiety coming from reduced sulfur compounds. Mutants Sat_1^-, Sat_3^- to Sat_6^-, and $Sat_7^-R_1$ lack enzyme fraction S, which is assumed to act at this point in thiosulfate formation.

The S moiety of thiosulfate is readily converted to reduced sulfur compounds in *Chlorella* (16), as shown in Figure 2. The SO_3 moiety of thiosulfate is rapidly converted to sulfate, as shown (16, 17).

Mutant Sat_2^- accumulates homocysteic acid and thiosulfate. It is thought that homocysteic acid arises from the SO_3 moeity of thiosulfate by transfer to the appropriate C_4 precursor. One possibility (of many) is that an excess of C_4 acceptor exists in Sat_2^- because of control problems in the main line. If this mutation is

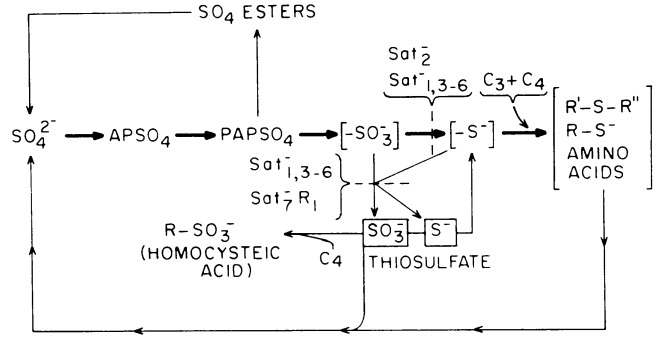

FIG. 2. A hypothetical scheme for sulfate reduction in *Chlorella*, emphasizing early reactions and showing the inferred metabolic defect(s) of various mutants.

between control points in the pathway, it is possible that the production of carbon skeletons for sulfur amino acid formation remains high even though no reduced sulfur is actually emerging from the blocked main line pathway. These carbon skeletons might then be available to react with the SO_3 moiety of thiosulfate (or, perhaps with ($—SO^-_3$)) to form homocysteic acid rather than the normally occurring homocysteine. Ragland and Liverman demonstrated that *Neurospora* could utilize a C_3 sulfonic acid, cysteic acid, as a sulfur source (27).

The accumulation of thiosulfate by mutant Sat_2^- requires special comment since this mutant readily utilizes exogenous thiosulfate for growth. Either this mutant keeps a high steady state concentration of thiosulfate by rapid synthesis to compensate breakdown or it is able to distinguish endogenous from exogenous thiosulfate. One mechanism of achieving the latter situation might be a thiosulfate transport system at the cell membrane which dismutes exogenous thiosulfate to oxidized and reduced moieties as they enter the cell. We would assume that this system would not act on endogenous thiosulfate.

Without genetic analysis one cannot tell which phenotypes are due to the same genes. The minimal number of genes, however, to explain our findings with these *Chlorella* phenotypes is two, which we can call α and β. Sat_1^-, and Sat_3^- to Sat_6^- are thought to be double mutants for reasons already presented; they would, therefore, be mutated in both α and β. $Sat_7^-R_1$ would represent reversion of gene α, let us say, and would thus be a mutant in the β locus. Sat_2^-, then, would represent a single mutation in gene α. Gene β would code for fraction S protein; while gene α would affect some activity in the main line between ($—SO_3^-$) and ($—S^-$) which has not yet been identified.

While we feel that many identifications of sulfite in the past may have been due to the use of methods which could produce sulfite as an artifact, if sulfite is indeed formed it could arise either from thiol-mediated reductive dismutation of thiosulfate (to yield sulfite from the SO_3 moiety) or from cleavage of ($—SO_3^-$). In radioactive experiments it could also arise from exchange of added nonradioactive sulfite with one of these species.

Our finding that mutant Sat_7^- is unable to form thiosulfate but reduces sulfate to the amino acid level has compelled the major features of the scheme which is presented. Thiosulfate on its side paths seems to provide a readily utilized source of sulfur at the oxidized and reduced levels and may serve as a sulfur store. In agreement with this, mutant Sat_7^- which cannot form thiosulfate has a rather long lag in growth on sulfate.

Interpreted in this way, there seems to be reasonable agreement about the outlines of the pathway for assimilatory sulfate reduction derived from the data of various laboratories and from studies of different organisms.

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