STUDIES ON SULFATE UTILIZATION BY CHLORELLA PYRENOIDOSA USING SULFATE-S^{35}; THE OCCURRENCE OF S-ADENOSYL METHIONINE[1,2]

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There is an abundance of nutritional evidence that higher plants and many microorganisms can use sulfate as the sole source of sulfur, but the mechanism of sulfate reduction and incorporation into organic compounds is not established. Horowitz and co-workers (9, 10, 17, 28) have investigated the intermediates of sulfate reduction in mutants of *Neurospora crassa* and have proposed cysteic acid and cysteine sulfinic acid as intermediates between sulfate and cysteine. On the basis of newer evidence which indicates that cysteine sulfinic acid is equivalent to cysteic acid and sulfite in Neurospora auxotrophs, Horowitz (11) has revised these earlier proposals. With the finding that all Neurospora mutants which are blocked between thiosulfate and cysteine can utilize elemental sulfur, Horowitz now suggests that the reductive pathway is a completely inorganic one leading from sulfate through sulfite, thiosulfate and sulfide (since Neurospora spores convert elemental sulfur to sulfide) to the linkage of sulfide to a three carbon acceptor to form cysteine. Cysteine sulfinic acid is probably oxidized to cysteic acid which is then converted to sulfite for further reduction. Singer and Kearney (25) have pointed out, however, that in systems that metabolize cysteine-sulfinic acid, the oxidation of this compound to cysteic acid is a very slow reaction or is entirely absent. They speculate that the site of linkage of inorganic sulfur to a carbon chain may be through the reaction of sulfite and pyruvate to yield beta-sulfinyl pyruvate.

Cowie and his co-workers (4) have shown that cysteic acid does not compete with the incorporation of radioactive sulfate in *Escherichia coli* and, therefore, may not be an intermediate in sulfate reduction in this organism. Ragland and Liverman (18) are currently investigating the intermediary metabolism of sulfate reduction in sulfur auxotrophs of Neurospora.

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The status of cystathionine as an intermediate between cysteine and methionine has also been investigated. While Horowitz et al have found this compound in certain auxotrophs of Neurospora (28), McRorie and Carlson (16) have indicated that there may be a route of conversion in Neurospora which does not involve cystathionine. Cowie has been unable to demonstrate cystathionine as an intermediate in *E. coli* by his competition techniques (4), but Lampen (12) has shown that auxotrophs of *E. coli* can utilize cystathionine as a sulfur source.

The reduction of sulfate to the level of hydrogen sulfide is highly endergonic and requires 8 electrons. By analogy to other oxidation-reduction systems one might suppose that the reduction would proceed in four steps with the transfer of two electrons per step. It is entirely possible that the inorganic species are not reduced directly, but after linkage to some carrier (such as adenylic acid) which changes the potential enough to permit reduction by compounds which ordinarily could not bring about the reaction. The free energy change in the reduction of sulfate by hydrogen may be calculated from the free energies of formation of the compounds from their elements (13):

$$S + O_2 \longrightarrow SO_4^= \qquad \Delta F = -176.1 \text{ kg Cal.}$$

$$S + H_2 \longrightarrow H_2S \text{ (aq.)} \qquad \Delta F = -6.52 \text{ kg Cal.}$$

Rearranging gives:

$$SO_4^= + H_2 \longrightarrow H_2S \text{ (aq.)} + O_2 \qquad \Delta F = +169.6 \text{ kg Cal.}$$

If one assumes that a reductant at the level of triphosphopyridine nucleotide is involved one may write (6):

$$SO_4^= + 4\,TPNH + 6\,H^+ \longrightarrow H_2S + 4\,H_2O + 4\,TPN^+ \qquad \Delta F = -208 \text{ kg Cal.}$$

If the reduction proceeded in this way, the process would be highly wasteful as far as energy is concerned. It is possible that the real reductant in the cells is closer to the potential of the sulfate-hydrogen sulfide system and that prior linkage of the inorganic species occurs to change the potentials of the sulfate —H_2S system.

Quite recently, in this connection, compounds of adenylic acid or adenosine with sulfur compounds have come into prominence. Hilz and Lipmann (7)

have described an enzyme system which brings about the activation of sulfate with ATP to yield adenosine-3′-phosphate-5′-phosphosulfate or "active sulfate" (19, 20). They have found this system in *Neurospora crassa* and in mammalian systems. Bandurski (1) has also described a similar system from yeast. The mammalian system also contains enzymes which bring about the reaction of "active sulfate" and *p*-nitrophenol to yield *p*-nitrophenol sulfate (7). Schlenk and his co-workers (23) have described a compound from yeast, thiomethyladenosine, which serves as a source of the thiomethyl group in other compounds and Schwartz and Shapiro (24) have shown that thiomethyl-adenosine serves as a source of the thiomethyl group of methionine in a mutant of *Aerobacter aerogenes*. Cantoni has demonstrated the formation of a similar compound, S-adenosyl methionine, from ATP and methionine in mammalian systems (2) which serves as a transmethylating agent, and is formed through a unique triphosphate cleavage of ATP which yields orthophosphate and pyrophosphate (3).

Nutritional studies which we have reported previously (21, 22) have shown that sulfate or cysteine will serve as the sole sulfur sources for the growth of Chlorella. Methionine apparently cannot serve as a sole sulfur source and leads to lower growth rates and chlorotic cells. We have also described experiments with methionine-S^{35} which have indicated that methionine enters the cells but that the sulfur label does not spread to compounds other than the methionine of protein to any appreciable extent. In these experiments difficulties were encountered in obtaining reproducible chromatographic patterns. The work to be described here attempts to establish conditions for radiosulfate utilization by Chlorella which are controllable and which lead to consistent chromatographic patterns.[4]

Materials and Methods

Chlorella pyrenoidosa Chick, obtained from Professor William Stepka, was grown on a modification of the medium of Hopkins (8) shown in table I. The medium was autoclaved for 40 minutes at 15 lbs/in^2. Growth took place in 2-liter flat-bottomed flasks for one to two weeks with mild aeration in a dark, temperature controlled room maintained at 26° ± 2° C; the cells were then harvested by centrifugation at 2° C, washed with cold sulfur-free medium and were resuspended in sulfur-free medium to give a final concentration of 0.1 ml of packed cells per ml. This suspension was then aerated for one half hour at room temperature before use.

One-ml aliquots were transferred to 12-ml centrifuge tubes, each equipped with a capillary aerator,

[4] At a time when the work to be described here had been completed, studies on sulfate utilization by Chlorella in the light were reported in abstract by Zweig and Hood (31). Liverman and Ragland (14) have recently reported investigations of the sulfate metabolism of the Alaska pea.

Table I

Medium for Growth of Chlorella

Substance	M/L	ml/L
KNO_3	0.01	
$MgNO_3$	0.005	
KH_2PO_4	0.004	
K_2HPO_4	0.001	
Na_2SO_4	0.005	
Ferric tartrate	1.8×10^{-5}	
Glucose	0.083	
Sodium citrate	0.0031	
Special A_5 solution *		1.0

* The constitution of Special A_5 solution is as follows: 500 ppm boron, 500 ppm manganese, 50 ppm zinc, 20 ppm copper, 50 ppm molybdenum, and 10 ppm cobalt, as the sulfur-free salts.

"Sulfur-free" medium is the above medium with Na_2SO_4, ferric tartrate, sodium citrate and glucose omitted.

for the various experiments in which radioactive sulfate was given. All experiments were carried out under ordinary laboratory lighting conditions of 25 ft-c or less.

At the end of the experimental period, the tubes were cooled in an ice bath, the cells were separated by centrifugation and were washed with 1.0 ml of cold sulfur-free medium. They were then suspended in 1.0 ml of either 1 *N* HCl or 0.1 M sodium citrate-citric acid buffer pH 5.5 and were subjected to three consecutive cycles of freezing and thawing between −10° C and room temperature. The cell debris was centrifuged and the extract removed. The debris was then washed with another aliquot of the extracting solution and was combined with the first extract. This constitutes the "acid soluble" (AE) fraction. In one experiment the cell debris was extracted once more with boiling 80 % aqueous ethanol (v/v) followed by 30 % aqueous ethanol (v/v) and then water at room temperature. When combined these constitute the "ethanol soluble" (EE) fraction. In some experiments the cell debris containing the material insoluble in HCl was subjected to acid hydrolysis in 2.0 ml of 6 *N* HCl for four hours at 108° C. The mixture after hydrolysis constitutes the "hydrolyzable material" (HE).

Chromatography of the extracts was carried out on washed sheets (57 × 46 cm) of Whatman no. 3 filter paper. Development was by solvent descent in two different mixtures:

1st Direction: The solvent of Mason and Berg (15) was employed:

methanol (absolute)	120 ml
n-butanol	60 ml
benzene	60 ml
water (distilled)	60 ml

2nd Direction: Stepka's solvent (26) was used:

n-butanol	100 ml
water (distilled)	50 ml
acetic acid (glacial)	22 ml

Radioautography was carried out in the usual manner as described previously (21).

Paper electrophoresis employed the conditions described by Hilz and Lipmann (7). Sodium citrate-citric acid buffer pH 5.5, 0.1 M was used with a migration path of 48 cm. The separations were run either for 17 hours at 190 V at 2° C or for four hours at 300 V at 5 to 10° C.

All counting for radioactive experiments was carried out at infinite thinness with a Nuclear model 163 scaler equipped with a 1.4 mg/cm^2 mica end-window tube. Sulfate-S^{35} was obtained from Oak Ridge National Laboratory as the carrier-free isotopic compound in dilute HCl.

Column chromatography was carried out with Dowex-50 as the adsorbent as described by Tabachnick and Tarver (27). Concentrations of HCl up to 6 *N* were employed instead of the maximum of 3 *N* used by these authors and different volumes of effluent were collected in some instances.

Iodoacetamide was synthesized by a method based on the brief description given by von Braun (29).

The enzyme used in the assay of "active sulfate" was prepared by a modification of the method of de Meio et al (5). Livers from four adult rats were homogenized in a glass homogenizer in three parts of ice-cold 0.1 M phosphate buffer pH 7.0. The homogenate was then subjected to centrifugation at 100,000 × G in a Spinco preparative centrifuge for 30 minutes. The supernatant was brought to one fourth saturation with ammonium sulfate and was then centrifuged for 10 minutes at 25,000 × G. Ammonium sulfate was added to the supernatant to bring it to one half saturation. The precipitate was isolated by centrifugation and was stored at − 10° C.

For assay purposes approximately 1 g of precipitate was dissolved in 10 ml of 0.1 M phosphate buffer pH 7.0 and was dialyzed against 0.005 M phosphate buffer pH 7.0 for 24 hours, at 2° C. The contents of the dialysis sack were then used in the described experiments.

Chlorella extract for the assay of "active sulfate" was prepared as follows: a suspension of 5 g wet weight of Chlorella was suspended in sulfur-free medium as described in materials and methods. To the suspension under aeration was added enough sodium sulfate to bring the final concentration to 0.005 M. Exactly three minutes after sulfate addition enough iodoacetamide was added to bring the suspension to 10^{-2} M iodoacetamide. Aeration was continued for 20 minutes. The suspension was then chilled in an ice bath and centrifuged. After washing with sulfur-free medium, the cells were suspended in 10 ml of 0.1 M phosphate buffer, pH 7.1, containing 0.5 M cysteine/ml in order to conjugate any excess iodoacetamide, and was subjected to three consecutive cycles of freezing and thawing. This extract constitutes the "Chlorella preparation."

Experimental Results

In contrast to the slow spread of the sulfur label from methionine-S^{35} to other compounds of the acid extracts of Chlorella (21), the spread of carrier-free sulfate-S^{35} is quite rapid. One tenth of a milliliter of packed Chlorella cells can remove over 10^6 cpm of radioactive sulfate from the medium and can convert it to reduced form in less than 15 minutes. The rapidity of the sulfate incorporation process necessitated some means of control which would stop the incorporation at various stages. If sulfhydryl compounds were produced from sulfate reduction, these could react with each other oxidatively during extraction and chromatography to form all the possible disulfides and other oxidative artifacts. In order to obviate both of these experimental difficulties, iodoacetamide was used in subsequent experiments. Iodoacetamide should act in at least two ways. If any compounds of a sulfhydryl nature were formed during sulfate incorporation, iodoacetamide should react with these to produce the corresponding S-substituted acetamides which could not form oxidative artifacts of the disulfide type. Secondly, if any of the enzymes involved in sulfate reduction required free sulhydryl groups for activity, iodoacetamide might be expected to inhibit the process to some extent.

The effect of iodoacetamide at various concentrations on radiosulfate uptake into the various fractions is shown in table II. Increasing concentrations of iodoacetamide serve to increase the incorporation of the label into the acid solubles (AE) at the expense of the acid insolubles (HE). The alcohol soluble material (EE) is a small part of the total and does not change significantly with increasing iodoacetamide concentration. Iodoacetamide was added five minutes after radioactive sulfate in these experiments.

Table II

Effect of Iodoacetamide Concentration on Sulfate Incorporation

Radioautograph no.	Iodoacetamide conc (M/L)	cpm/0.1 ml cells			
		AE	EE	HE	Total recovered
		× 10^{+5}	× 10^{+5}	× 10^{+5}	× 10^{+5}
1-15	0	2.9	0.28	6.0	9.3
2-15	10^{-5}	2.8	0.28	4.5	7.6
3-15	10^{-4}	3.8	0.32	5.2	9.3
4-15	10^{-3}	5.2	0.17	4.3	9.6
5-15	10^{-2}	5.9	0.26	3.5	9.7

Each tube contained 0.1 ml volume of Chlorella cells in 1.0 ml sulfur-free medium. Each received 12 × 10^{+5} cpm $S^{35}O_4^=$ at zero time. Iodoacetamide was added 5 min after the radioactive sulfate. Tubes were incubated for 1 hr and extracted with HCl. Incorporation into the acid solubles (AE), ethanol solubles (EE) and insolubles (HE) is shown.

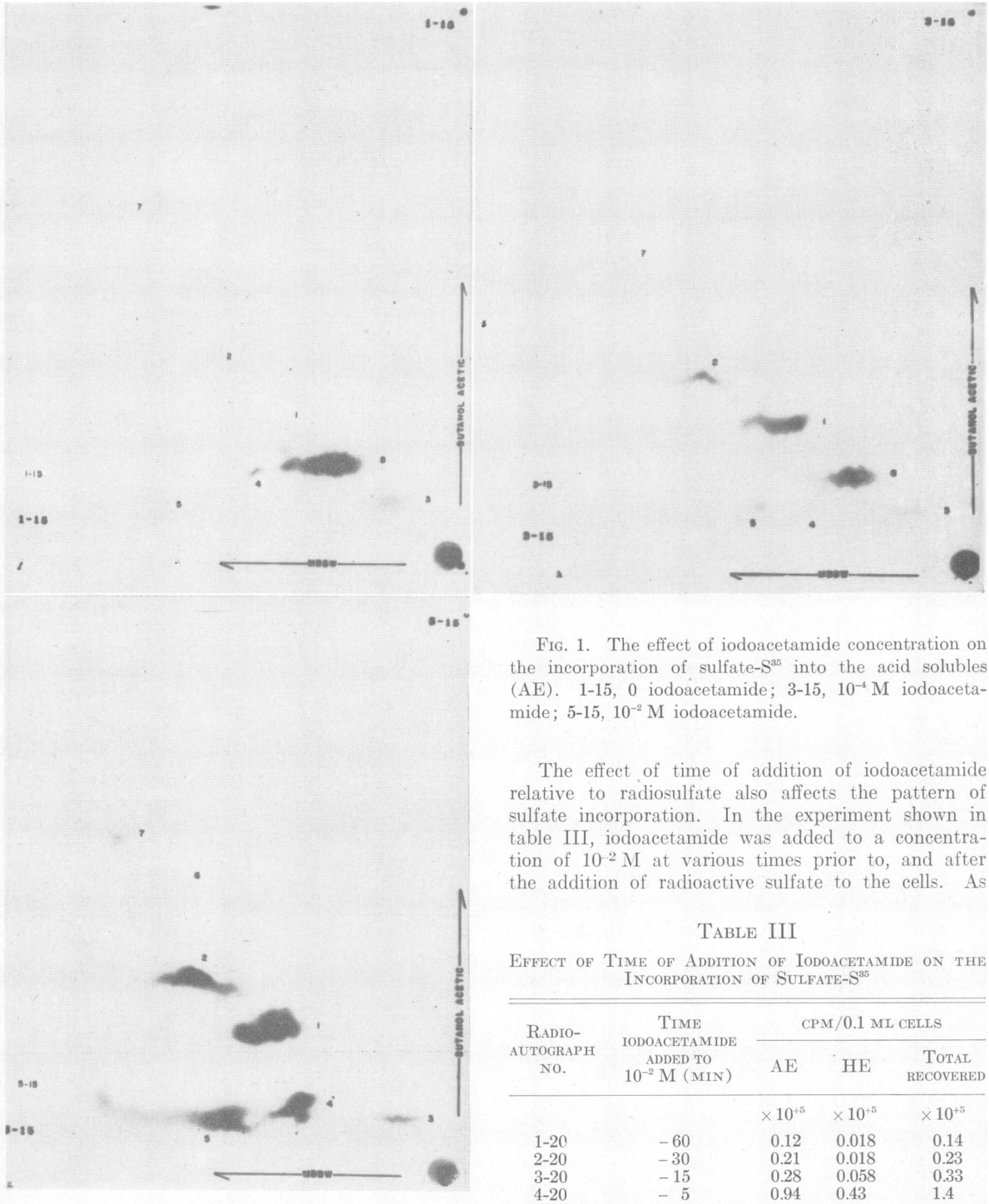

FIG. 1. The effect of iodoacetamide concentration on the incorporation of sulfate-S^{35} into the acid solubles (AE). 1-15, 0 iodoacetamide; 3-15, 10^{-4} M iodoacetamide; 5-15, 10^{-2} M iodoacetamide.

A comparison of the radioautographs of the acid extracts (AE) at various iodoacetamide concentrations shown in figure 1 reveals the disappearance of compound 8 with the concomitant appearance of several other compounds as the iodoacetamide concentration is increased.

The effect of time of addition of iodoacetamide relative to radiosulfate also affects the pattern of sulfate incorporation. In the experiment shown in table III, iodoacetamide was added to a concentration of 10^{-2} M at various times prior to, and after the addition of radioactive sulfate to the cells. As

TABLE III

EFFECT OF TIME OF ADDITION OF IODOACETAMIDE ON THE INCORPORATION OF SULFATE-S^{35}

RADIO-AUTOGRAPH NO.	TIME IODOACETAMIDE ADDED TO 10^{-2} M (MIN)	CPM/0.1 ML CELLS		
		AE	HE	TOTAL RECOVERED
		$\times 10^{+5}$	$\times 10^{+5}$	$\times 10^{+5}$
1-20	−60	0.12	0.018	0.14
2-20	−30	0.21	0.018	0.23
3-20	−15	0.28	0.058	0.33
4-20	− 5	0.94	0.43	1.4
5-20	0	9.0	5.9	15.0
6-20	10	5.0	8.8	14.0
7-20	No iodoacetamide	4.1	9.1	13.0

Each tube contained 0.1 ml volume of Chlorella cells in 1.0 ml of sulfur-free medium. Each received 17×10^5 cpm $S^{35}O_4^=$ at zero time. Tubes were incubated for 1 hr after $S^{35}O_4^=$ addition. HCl was the extracting solution.

TABLE IV

INCUBATION OF CHLORELLA EXTRACT WITH RAT LIVER ENZYMES

TUBE NO.	CHLORELLA PREP	RAT ENZYMES	VERSENE 0.5 M	Mg 0.2 M	ATP 0.1 M	$SO_4^=$ 0.15 M	NITROPHENOL SULFATE FORMED *
	ml	*ml*	*ml*	*ml*	*ml*	*ml*	*μg*
1	3.0	...	...	...	...	...	17
2	3.0	1.0	...	...	...	...	56
3	3.0	1.0	0.3	...	...	...	58
4	...	1.0	...	...	...	...	0
5	...	1.0	...	0.1	0.1	0.3	80
6	...	1.0	...	...	0.1	0.3	6
7	...	1.0	0.3	0.1	0.1	0.3	2

All tubes contained 1.0 ml phosphate buffer (0.1 M, pH 7.1), 100 μg *p*-nitrophenol and enough distilled water to bring to 5.5 ml.

* The formation of nitrophenol sulfate was measured by the disappearance of the color due to *p*-nitrophenol after a correction for turbidity.

may be seen from 1-20 to 4-20 in table IV, iodoacetamide added prior to the addition of radioactive sulfate almost completely inhibited the uptake of sulfate by the cells. The simultaneous addition of iodoacetamide and radiosulfate (5-20) permitted a large uptake of the radiosulfate into both the acid solubles (AE) and the insolubles (HE) as did iodoacetamide added 10 minutes after radiosulfate (6-20).

The radioautographs of the acid solubles under these conditions are shown in figure 2. As may be seen from radioautograph 4-20 in figure 2, iodoacetamide added five minutes prior to radiosulfate has inhibited the spread of the label from sulfate to other compounds of the acid extracts. The same situation is found for the simultaneous addition of radiosulfate and iodoacetamide; in this case even though the

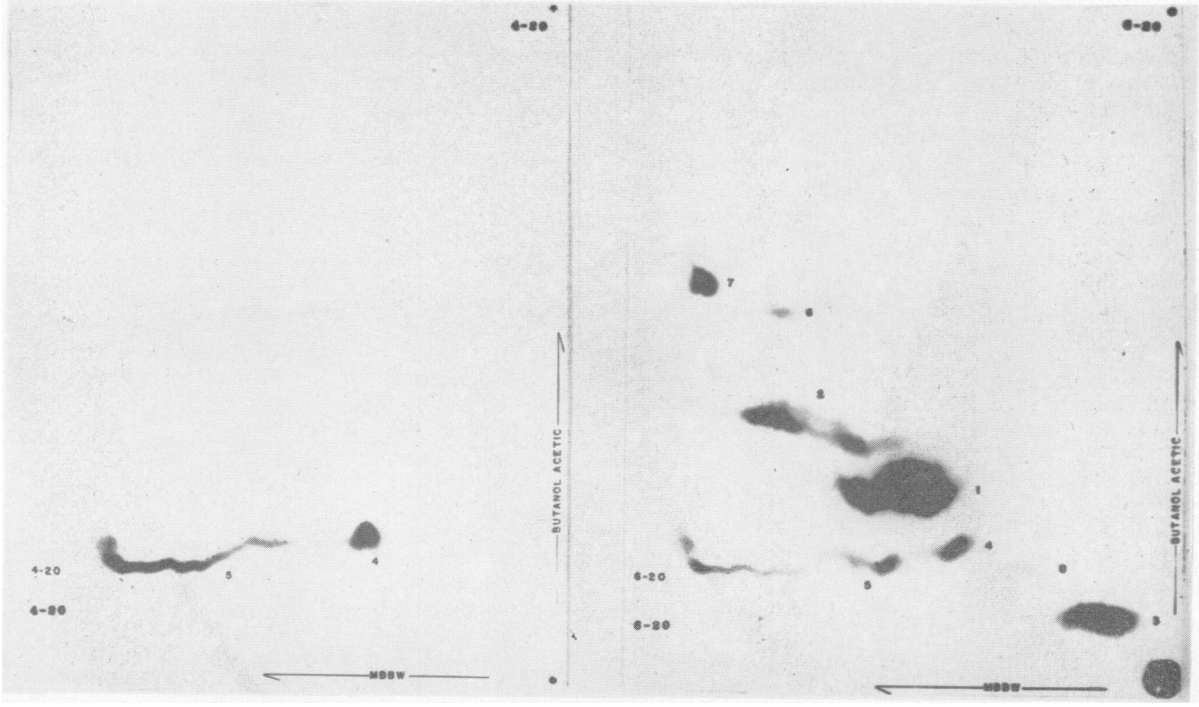

FIG. 2. The effect of time of addition of iodoacetamide relative to sulfate-S^{35} on the incorporation of sulfate-S^{35} into the acid solubles (AE). 4-20, iodoacetamide added 5 minutes prior to radiosulfate; 6-20, iodoacetamide added 10 minutes after radiosulfate.

spread of sulfate into the other compounds of the acid extracts (AE) is inhibited, there is still a considerable incorporation of label into the insolubles (HE). If iodoacetamide is added 10 minutes after the addition of radioactive sulfate, the same radioautographic pattern is found as was obtained in the previous experiment for the addition of iodoacetamide five minutes after the addition of radiosulfate. (Compare figure 1, 5-15 and figure 2, 6-20.)

The effect of iodoacetamide addition between 0 and 10 minutes after radiosulfate was explored using paper electrophoresis to separate the compounds of the acid solubles (AE). If iodoacetamide is added three minutes after the addition of radioactive sulfate, the pattern of compounds is different from that found for the addition of iodoacetamide five minutes after the isotopic compound. Since the pattern found at five minutes is the same as that found at 10 minutes, the spread of radioactivity into the acid extracts (AE) from radiosulfate is probably complete by five minutes after the addition of radiosulfate to the cells.

One of the compounds found on the three-minute electrophoretograms had a high mobility towards the anode. The possibility arose that this compound might be an "active" sulfate as described by Hilz and Lipmann (7). A cross incubation between a rat liver enzyme system in which the activating enzyme had been inactivated with versene, and an extract of Chlorella which received iodoacetamide three minutes after radiosulfate was carried out to test this possibility. The transferase in the liver extract should catalyze the reaction of *p*-nitrophenol with any "active" sulfate in the Chlorella extract to yield *p*-nitrophenol sulfate. The results of such an experiment are shown in table IV.

The tubes which contained the Chlorella preparation alone (tube 1), the rat liver enzyme alone (tube 4), the complete rat enzyme system without magnesium (tube 6), and the complete rat enzyme system with added magnesium and versene (tube 7) failed to produce a significant amount of nitrophenol sulfate. The Chlorella preparation plus the unfortified rat enzyme system (tube 2) produced a significant amount of nitrophenol sulfate, as did the same combination with added versene (tube 3). The largest formation of nitrophenol sulfate was observed in the completely fortified rat liver enzyme system (tube 5). The results suggest that a compound similar to the "active" sulfate found in rat liver is present in Chlorella during sulfate utilization. Actual proof will have to await studies on cell-free enzyme systems of Chlorella.

IDENTIFICATION OF S-ADENOSYL METHIONINE: In attempts to identify the radioactive compounds produced from radiosulfate utilization, indirect methods of chromatography and electrophoresis were employed since the amount of compounds isolated was small. Evidence obtained from paper electrophoresis, and ion-exchange chromatography on Dowex-50 indicates that compounds of several degrees of acidity and basicity are present in the acid extracts (AE). A description of the separation of the radioactive compounds of the acid extracts on Dowex-50 is shown in table V.

Only one of the compounds present in the acid extracts has been identified with any certainty. This is compound 3 shown in figure 2. Compound 3 was suspected to be quite polar due to its low R_f in both solvent systems employed. This was confirmed by chromatography on Dowex-50 and paper electrophoresis where it behaved as a fairly strong base. If one assumed that the basicity of the molecule was due to the sulfur atom(s) which it contained then a sulfonium configuration suggested itself. When the amount of extract placed on the chromatographs was greatly increased, a quenching spot under light of approximately 260 mμ was observed to be coincident with the radioactivity from spot 3. These properties suggested that the compound might be S-adenosyl methi-

TABLE V

SEPARATION OF S^{35}-LABELED COMPOUNDS ON DOWEX-50

FRACTION NO.	NORMALITY OF HCl USED TO ELUTE	ML HCl COLLECTED PER FRACTION	CPM IN TOTAL FRACTION **	COMPOUNDS PRESENT *	EXAMPLES OF KNOWN COMPOUNDS SEPARATED BY TABACHNIK AND TARVER (19)
			$\times 10^{+5}$		
D-1	0.0	125	1.59	2, 4, 5	Taurine, cysteic acid
D-2	1.5	112	1.24	2	.....
D-3	2.5	615	0.86	8, 1 (?)	Glutathione, methionine, methionine sulfoxide, cystine, cystathionine
D-4	4.0	150	0.08	.....	.....
D-5	4.0	180	0.06	.....	.....
D-6	6.0	190	0.27	3	.....
D-7	6.0	160	0.08	.....	.....

* The numbers refer to the spots found on subsequent paper chromatography of these fractions in MBBW, butanol-acetic. The numbers are the same as given in Figure 2.

(?) Three compounds now occupy the place on the paper formerly occupied by only one.

** CPM placed on the column at the start: 5.15×10^6; total recovered: 4.18×10^5.

onine which had been described from mammalian systems by Cantoni (2). An authentic sample of S-adenosyl methionine kindly provided by Dr. Cantoni, showed exact co-chromatography with spot 3 and a similar electrophoretic mobility. It has been shown that S-adenosyl methionine yields thiomethyladenosine on mild hydrolysis (2). Mild hydrolysis of spot 3 yielded a compound whose R_f values were similar to those of an authentic sample of thiomethyladenosine in our solvent systems. Exact co-chromatography of known compounds of biological importance, other than S-adenosyl methionine with the unknown spots has not been realized. Partial coincidence of some compounds has been observed suggesting that some of the unknown spots may contain more than one compound. For example, spot 1 partially co-chromatographs with synthetically prepared S-cysteine acetamide. It is possible that spot 1 also contains the acetamide derivatives of closely related sulfhydryl compounds such as homocysteine or glutathione. It is hoped that in the future the application of a combination of techniques of separation will permit the further identification of these compounds.

Discussion and Conclusions

We have attempted to establish conditions which would lead to control of the rapid process of sulfate utilization and which would yield reproducible chromatographic patterns of compounds that are formed from sulfate. This has been realized through the employment of relatively mild extraction techniques and by the use of iodoacetamide as an inhibitor of the sulfate reduction process. It was found that the simultaneous addition of iodoacetamide and radiosulfate to the cells results in a large uptake of label but the radioactivity found in the acid extracts under these conditions remains largely as sulfate. If iodoacetamide is added prior to radiosulfate, the uptake of label is strongly inhibited. This evidence suggests that at least one site of iodoacetamide inhibition occurs very early in the metabolism of sulfate; perhaps the site of inhibition may be the activating system itself. The activity of the sulfate activating system of mammalian tissues is inhibited by O-[(3 hydroxymercuri-2-methoxypropyl)] carbamyl phenoxyacetic acid; this inhibition is reversed by cysteine (7). Studies of our own indicate that the sulfate activating system of mammalian systems is sensitive to iodoacetamide (Whitehouse, M. and Schiff, unpublished). It is possible, therefore, that the sulfate activating system of Chlorella is sensitive to iodoacetamide and that this system is the site of inhibition observed in the experiments described above. An equally likely possibility to be considered is that iodoacetamide is merely inhibiting the systems of energy production within the cell which is thereby deprived of an energy source for active accumulation of sulfate.

Although the simultaneous addition of radiosulfate and iodoacetamide inhibits the spread of label into other compounds of the acid extracts, considerable label is still incorporated into the insoluble fraction which has not yet been fractionated. The label in this fraction may reside only in sulfate conjugates of polysaccharides and other large molecules. If the label should be present in reduced form such as the amino acids of protein, however, this would indicate that there may be a route of sulfate reduction in Chlorella which does not involve the compounds of the acid extracts.

The mechanism by which S-adenosyl methionine is formed in Chlorella may be different from the activating system described by Cantoni from mammalian systems (2). In our experiments reported previously (21) exogenous methionine was not utilized appreciably by Chlorella to form compounds of the acid extracts. The experiments described in this paper show that adenosyl methionine is formed during the reduction of sulfate by this organism. It would be tempting to visualize S-adenosyl methionine as one of the last compounds in a chain beginning with "active sulfate" and leading, perhaps, through a series of sulfur conjugates of AMP to more reduced sulfur compounds. Bandurski has recently reported a system which is capable of releasing pyrophosphate from ATP in the presence of inorganic sulfite (30). The compound of adenylic acid and sulfite which one would expect has proven to be too unstable to be isolated. Although this compound is very unstable, it could conceivably play a part in such a chain of reduction products of sulfate. It is also interesting to note that the sulfate activating system has been found in yeast and in Neurospora both of which are capable of reducing inorganic sulfate. The sulfate activating system in mammalian cells, which are incapable of reducing sulfate, is accompanied by a phenol transferase system while the phenol transferase system is apparently absent from Neurospora which is capable of carrying out the reduction process.

Although many spots corresponding to compounds formed from radioactive sulfate are found on the reported chromatograms, the only compound which we have identified with any certainty is S-adenosyl methionine. It is hoped that the other compounds found will be identifiable in future studies and that the elucidation of their role in the intermediary metabolism of sulfate will similarly be possible.

Summary

Sulfate-S^{35} is taken up rapidly by *Chlorella pyrenoidosa* and is converted to reduced compounds within the cell. One of the compounds formed from $S^{35}O_4^=$ shows the properties of S-adenosyl methionine. Preliminary evidence is also presented for the formation of "active" sulfate. Techniques have been developed to stabilize and control the process of sulfate reduction by Chlorella by using iodoacetamide as an inhibitor. These techniques are described.

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