Thiosulphate Metabolism and Rhodanese in *Chromatium* sp. strain D

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(Received 27 August 1965)

SUMMARY

Suspensions of *Chromatium* D oxidized thiosulphate completely to sulphate (in the light) under anaerobic conditions in the presence of carbon dioxide. During the oxidation, intracellular sulphur accumulated transiently and sulphate production followed a biphasic pattern. Using inner-³⁵S-thiosulphate, the initial burst of sulphate production, which accounted for about half of the total yield, occurred principally at the expense of the inner (SO₃-) atom of thiosulphate; there was no intracellular accumulation of labelled sulphur. The radioactivity from outer-³⁵S-thiosulphate accumulated transiently within the organisms and was transferred to sulphate at a rate which was similar to the second phase of sulphate production; most of the outer (S-) atom therefore passed through the stage of endogenous sulphur. Extracts of the organism catalysed the cyanolysis of thiosulphate to give sulphite and thiocyanate. An assay for this enzyme, rhodanese, based on the spontaneous reduction of 2,6-dichlorophenol-indophenol by sulphite was developed. The enzyme was also detected in extracts of *Athiorhodaceae*. The enzyme was partially purified from extracts of *Chromatium* D and resolved into two active fractions. It is concluded that endogenous sulphur is an intermediate in the oxidation of thiosulphate by *Chromatium* D and that the cleavage of the S—S bond in the molecule is a key step in the oxidation process. Rhodanese, which catalyses this type of reaction, may be concerned in this cleavage.

INTRODUCTION

Photosynthetic sulphur bacteria assimilate carbon dioxide into cell material under anaerobic conditions in the light with the concomitant oxidation of a variety of reduced inorganic sulphur compounds to sulphate or to an intermediate oxidation product such as sulphur (van Niel, 1931; Larsen, 1952, 1953). The ability of purple sulphur bacteria (*Thiorhodaceae*) to accumulate globules of sulphur intracellularly during autotrophic growth on sulphide and thiosulphate is a characteristic feature of these organisms (van Niel, 1931, 1936; Eymers & Wassink, 1937). The role of endogenous sulphur in the oxidation of thiosulphate by *Thiorhodaceae* is uncertain.

The oxidation of inorganic sulphur compounds has been studied more intensively with thiobacilli which oxidize them under aerobic conditions in the dark to sulphate or intermediate oxidation products such as polythionates and elementary sulphur. The accumulation of extracellular sulphur during such processes has been variously attributed to biological reactions (Starkey, 1935; Peck, 1960) and to spontaneous

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reactions (Tamiya, Haga & Huzisige, 1941; Vishniac, 1952; Rittenberg & London, 1964). The results of experiments both with whole organisms and with extracts has led to two differing schools of thought concerning the mechanism of the initial attack on thiosulphate:

(1) the oxidative coupling of two molecules to give tetrathionate (Trudinger, 1958, 1959; Vishniac & Santer, 1957; Rittenberg & London, 1964);

(2) the reductive cleavage of one molecule to give sulphide and sulphite (Peck, 1960; Peck & Fisher, 1962).

In the present work, the metabolism of thiosulphate and other inorganic sulphur compounds has been studied in the purple sulphur bacterium *Chromatium* strain D to establish the mechanism of thiosulphate oxidation and the role of intracellular sulphur in this process. In the course of this work thiosulphate sulphur-transferase activity was demonstrated in extracts. This enzyme catalyses the cyanide-dependent cleavage of thiosulphate to form thiocyanate and sulphite (equation 1) and has been extensively studied with purified preparations from mammalian sources such as liver and kidney (Green & Westley, 1961; Westley & Nakamoto, 1962). In the presence of reduced lipoic acid ($\text{Lip}(\text{SH})_2$) the enzyme catalyses the reductive cleavage of thiosulphate (equation 2); lipoyl persulphide is an intermediate in this reaction (Villarejo & Westley, 1963*a*, 1963*b*).

Thiosulphate sulphur-transferase EC 2.8.1.1. (which will be referred to as rhodanese) has been partially purified from *Chromatium* D and its possible relevance to thiosulphate metabolism has been examined. This work has been briefly reported elsewhere (Smith, 1964, 1965).

METHODS

Organisms. The culture of *Chromatium* sp. strain D and its maintenance as stab cultures on the thiosulphate + salts medium have been previously described (Hurlbert & Lascelles, 1963). This organism was used throughout these studies except in the investigations with extracts where the Athiorhodaceae, *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* were also used. These organisms were grown anaerobically in the light on the malate + glutamate medium described by Lascelles (1959) fortified in the case of *Rhodospirillum rubrum* with yeast extract (0.2 %, w/v).

Media. The thiosulphate + bicarbonate + salts medium used for the growth of *Chromatium* D was that described by Hurlbert & Lascelles (1963). In some experiments organic substrates were used in place of thiosulphate, as described previously.

Growth of cultures. In preparing organisms for experiments with suspensions and cell-free extracts the inoculum was taken from a stab culture or from a liquid culture for volumes in excess of 1 l. (approximately 1 ml. of liquid culture/100 ml. of medium). The cultures were incubated in completely filled glass-stoppered bottles at 34° in a light cabinet fitted with banks of 15 W. tungsten lamps. The organisms were free of endogenous sulphur and fully grown after 2–4 days.

For the quantitative investigation of growth on various inorganic sulphur compounds, sulphide (0.01 %, w/v) and thiosulphate (up to 0.3 %, w/v) were omitted from the complete salts medium and replaced by the appropriate sulphur compound. The inoculum, taken from a liquid culture, gave an initial concentration equiv. 30 mg. protein/l.; for growth on endogenous sulphur, the initial concentration equiv. was 70 mg. protein/l. The inoculated medium was incubated in Roux bottles under oxygen-free nitrogen in a glass-sided water bath at 34° illuminated with 150 W. tungsten lamps 15 cm. from the bath; the light intensity was approximately 150 ft.c. at the surface of the vessel. The densities of the cultures were measured at the end of growth as protein determined by the biuret method described by Hurlbert & Lascelles (1963). The protein:dry weight ratios of organisms grown on various inorganic sulphur compounds were in the range 0.5–0.6.

Preparation and incubation of suspensions. Organisms were normally harvested from the thiosulphate + salts medium at the end of growth when largely free of endogenous sulphur by centrifuging at 2500 g for 15 min., washed twice in about one-fifth the original culture volume of 0.04 M-potassium phosphate buffer (pH 7.5) and resuspended in 0.1 M-potassium phosphate buffer (pH 6.75) containing sodium bicarbonate (60 mM) and sodium thiosulphate (2–40 mM). Suspensions were incubated at 34° and gassed continuously with N₂ + 5 % CO₂; the final pH was 7.3. For investigations at low intensities of illumination, suspensions containing up to 2.0 mg. protein/ml. were incubated in flat-sided glass bottles in a glass-sided water bath as described above. Incubations at higher intensities of illumination (up to 500 ft.c. at the level of the suspension) were made in a photosynthetic Warburg tank (American Instrument Co., Inc., Washington, D.C.) illuminated from below with a bank of twelve 100 W. tungsten lamps coupled to a rheostat. Suspensions containing 0.2–0.5 mg. of protein/ml. were incubated in this bath in flat-bottomed conical flasks shaken transversely at 100 cyc./min. with an amplitude of 4 cm.

Preparation of extracts. Organisms were normally harvested at the end of growth when largely free of endogenous sulphur by centrifuging at 2500 g for 15 min., washed twice in one-tenth the original culture volume of 0.04 M-potassium phosphate buffer (pH 6.1) and resuspended in the same buffer at concentrations to 50 mg. protein/ml. Crude extracts were prepared by the French press or by ultrasonic treatment (Hurlbert & Lascelles, 1963). They were centrifuged at 18,000 g for 20 min. to remove whole organisms and debris. Cell breakage and all subsequent fractionations were done at 0–5°; extracts and extract fractions were stored at –20°.

Assay for rhodanese. The reaction mixture contained, in a volume of 3 ml.: tris buffer (pH 8.7), 300 μ mole; sodium thiosulphate, 150 μ mole; half neutralized sodium cyanide (NaCN/HCl, 2.5/1., mole/mole), 150–240 μ mole; 2,6-dichlorophenol-indophenol (DCIP), 0.5 μ mole; *N*-methylphenazoniummethosulphate (PMS), 0.25 mg.; enzyme preparation equivalent to 0.5 mg. of crude extract protein. The reaction was initiated by the addition of cyanide to the otherwise complete reaction mixture. The reference cuvette contained the complete reaction mixture without thiosulphate. DCIP reduction was followed at room temperature (18–20°) using a recording double-beam grating spectrophotometer (Optica type CF4DR Optica U.K. Ltd., Gateshead-on-Tyne, England). The millimolar extinction coeffi-

cient of DCIP at 600 m μ is 19.1 (Basford & Huennekens, 1955). The unit of enzyme activity was defined as the amount of enzyme which catalysed the reduction of 30 μ mole of DCIP/min.

Purification of rhodanese. Particle-bound pigment was removed from the extract prepared from 12 l. of fully grown culture by centrifuging at 100,000 g for 60 min. in a Spinco model L ultracentrifuge. The supernatant fraction was treated with 5% (w/v) streptomycin sulphate (Glaxo Laboratories Ltd., Greenford, England) in 0.04 M-potassium phosphate buffer (pH 6.1) to a final concentration of 0.6 mg. of streptomycin sulphate/mg. extract protein. Precipitated nucleic acid was removed after 15 min. by centrifuging at 18,000 g for 20 min. Protein was precipitated from the supernatant fluid with saturated ammonium sulphate (pH 7.0) at a final concentration of 90% saturation (by volume). After 30 min., the precipitated protein was removed by centrifuging at 18,000 g for 20 min., and was redissolved in a minimum amount of 0.04 M-potassium phosphate buffer (pH 6.1). The concentrated protein fraction was applied to a column (35 $\times$ 1.5 cm.) of Sephadex G75 (Pharmacia) and eluted with 0.01 M-potassium phosphate buffer (pH 6.1). The enzyme recovered from the column was transferred to a column (10 $\times$ 1.5 cm.) of DEAE-DE 50 cellulose (Whatmans.). Protein was eluted with potassium phosphate buffer (pH 6.1) using a concentration gradient (10–500 mM); the flow rate was 2 ml./min. and the eluate was collected in 5 ml. fractions.

Analytical methods. With crude extracts, protein was precipitated and pigments were removed by heating the samples to 65° with 5 ml. of acid ethanol (400 ml. of ethanol, 5 ml. of glacial acetic acid and 100 ml. of water). The extraction procedure was repeated at least twice. The protein suspension was collected by centrifuging and the protein determination carried out as described by Hurlbert & Lascelles (1963). Protein determinations at all stages of extract fractionation after the removal of pigments from the crude extract were made by u.v. absorption (Layne, 1957).

The concentrations of thiosulphate and sulphate in suspensions were determined using samples of the supernatant fluid after the organisms had been removed by centrifuging. Thiosulphate was determined titrimetrically with standardized iodine, and by the methods of Schön (1959) and Sorbo (1957). Sulphate was determined by the method of Gleen & Quastel (1953).

An approximate estimate of the accumulation of sulphur within the organisms in a suspension oxidizing thiosulphate was made by following the change in turbidity of the suspension with an EEL photoelectric colorimeter with a neutral density filter (Evans Electroselenium Ltd., Halstead, Essex, England.).

Phosphate was determined by the method of Taussky & Shorr (1953).

Measurement of ^{35}S in the organisms and sulphate in suspensions. For determination of radioactivity in the organisms, samples of suspension containing 0.06 mg. protein were drawn through cellulose plastic filters under vacuum (Millipore filters of pore size 0.65 μ held in a Pyrex microanalysis filter-holder; Millipore Filter Corporation, Bedford, Mass., U.S.A.). The organisms on the filter were washed with water. Samples for the determination of ^{35}S in the sulphate were centrifuged to remove the organisms. Supernatant fluid containing up to 6 μ mole sulphate was made up to 9 ml. with water and mixed with 1 ml. of reagent (15 g. of barium chloride and 4.8 ml. of concentrated hydrochloric acid in 100 ml. of water); samples of the sus-

pension containing up to 0.7 mg. dry weight of barium sulphate were drawn through cellulose plastic filters under vacuum and washed with water.

The filter pads containing organisms and barium sulphate were dried at room temperature and glued to aluminium foil planchets. Samples were counted with an end-window Geiger-Müller tube (EHM25, General Electric Co., England) in a lead castle coupled to an Ecko Automatic Scaler (type N530D, Ecko Electronics Ltd., England). Self-absorption was negligible and the counts were corrected for background. The efficiency of counting was approximately 3.5%.

Chemicals. Tetrathionate was prepared by the method of Gilman (1946).

The sodium salts of inorganic sulphur compounds were used throughout these studies.

³⁵S-thiosulphates were obtained from the Radiochemical Centre (Amersham, Bucks., England). The original specific activities were in the range 10–20 mc./μmole.

RESULTS

Growth of Chromatium D

Autotrophic growth of the organism was supported by sulphide, sulphur, thiosulphate and sulphite (Table 1); intracellular sulphur accumulated transiently during growth on sulphide and thiosulphate and such endogenous stores of sulphur supported the further growth of the organism on the salts medium in the absence

Table 1. *Growth of Chromatium D on inorganic sulphur compounds*

The basal salts medium, containing 0.5% NaHCO₃, was supplemented with thiosulphate, sulphide, sulphite or tetrathionate, each at a final concentration of 4 mM. Organisms that were in the stationary phase and largely free of sulphur were used as the inoculum. In the experiment showing growth on endogenous sulphur, organisms were harvested during growth on thiosulphate when packed with sulphur and used as the inoculum for sterile sulphur-free salts medium (see Methods section). Another sample of these organisms was incubated in the suspension medium at a concentration of 1.5 mg. protein/ml. for several hours; the sulphate produced was determined and used as a measure of the intracellular sulphur. Growth was estimated as the increase in protein, when the cultures had reached the stationary phase.

Sulphur compound	Growth yield (mg. protein/mmmole)	Reducing capacity (electrons/molecule)
Thiosulphate	27	8
Sulphide	27	8
Sulphite	6	2
Sulphur (endogenous)	19	6
Tetrathionate	No growth	14
Tetrathionate + thiosulphate	No growth	—

of added sulphur compounds (Table 1). Tetrathionate did not support the autotrophic growth of the organism and the polythionate inhibited the growth of the organism on thiosulphate (Table 1). It also inhibited growth on organic compounds such as pyruvate and succinate.

Under autotrophic conditions the extent of growth was dependent on the amount of sulphur compound in the medium and, on a molar basis, was proportional to the number of electrons released during oxidation of the sulphur compound to sulphate (Table 1).

*Oxidation of inorganic sulphur compounds by suspensions of
Chromatium D*

Thiosulphate oxidation. Suspensions of inorganic grown organisms in phosphate buffer and bicarbonate, at pH 7.3, oxidized thiosulphate completely to sulphate under anaerobic conditions in the light (Fig. 1). The production of sulphate from thiosulphate followed a biphasic pattern. The rapid disappearance of thiosulphate was accompanied by the rapid production of up to 60 % of the theoretical yield of sulphate and the intracellular accumulation of sulphur. When thiosulphate had disappeared, sulphate was produced at a much slower rate, presumably from the endogenous sulphur which had accumulated during the initial period. Under these conditions, tetrathionate was not detected in suspensions during thiosulphate oxidation. The rates of thiosulphate disappearance and of sulphur accumulation were dependent on the intensity of illumination in the range 0–200 ft.c. Above the light saturation point, the rate of thiosulphate disappearance was strictly dependent on the density of the suspension. The kinetics of thiosulphate utilization and sulphate production by suspensions were, however, similar at both low (150 ft.c.) and high (500 ft.c.) intensities of illumination.

Table 2. *Thiosulphate oxidation by suspensions of organisms under various conditions*

Organisms grown on thiosulphate were harvested and resuspended to a density of 1.5 mg. protein/ml. in the suspension medium containing thiosulphate (2 mM). The suspension was incubated at a light intensity of 150 ft.c., with a gas phase of N₂/5 % CO₂ or air/CO₂.

Conditions	Thiosulphate disappearance (μ mole/hr/ml.)	Sulphate produced after 20 hr (% theoretical)
Anaerobic dark	0	0
Anaerobic light	2.9	96
Aerobic, dark	2.2	62
Aerobic, light	5.3	72

The oxidation of thiosulphate was not affected by the omission of phosphate, by the addition of organic compounds (pyruvate, 10 mM; succinate, 10 mM), or by the presence of considerable amounts of endogenous sulphur within the organisms of the suspension. Under anaerobic conditions, thiosulphate did not disappear in the absence of the physiological oxidant, bicarbonate, or in the dark. Under aerobic conditions (gas phase, air/CO₂), however, thiosulphate oxidation by suspensions was not light-dependent but was increased by illumination (Table 2).

Oxidation of other inorganic sulphur compounds. Suspensions of thiosulphate-grown organisms harvested during growth, when they contained considerable amounts of intracellular sulphur, oxidized this endogenous sulphur to sulphate under anaerobic conditions in the light. Sulphate production was accompanied by a fall in the turbidity of the suspension (Fig. 2). The fact that organisms oxidized endogenous sulphur to sulphate enabled an estimate to be made of the magnitude of sulphur accumulation during growth on thiosulphate. Determination of the sulphate produced by organisms harvested when they contained much intracellular

Fig. 1. Oxidation of thiosulphate by a suspension of *Chromatium D*. A suspension of thiosulphate-grown organisms containing 0.25 mg. of protein/ml. was incubated under anaerobic conditions at a light intensity of 500 ft.c. in the presence of thiosulphate (2 mM). Thiosulphate (— Δ —) disappearance, sulphur (— $\blacksquare$ —) accumulation and sulphate (— $\circ$ —) production were determined, as described in the Methods section.

Fig. 2. Oxidation of endogenous sulphur. A suspension of thiosulphate-grown organisms (0.25 mg. of protein/ml.) containing endogenous sulphur was incubated under anaerobic conditions at a light intensity of 500 ft.c. Sulphur (— $\blacksquare$ —) utilization and sulphate (— $\circ$ —) production were determined.

Table 3. *Oxidation of inorganic sulphur compounds by suspensions of organisms*

Suspensions of thiosulphate-grown organisms (1.5 mg. of protein/ml.) were incubated under anaerobic conditions at a light intensity of 150 ft.c. with the appropriate sulphur compound—sulphide (10 mM), sulphite (10 mM), thiosulphate (10 mM) or exogenous elementary sulphur. In the experiment showing the oxidation of endogenous sulphur, the organisms contained much intracellular sulphur when harvested. The rate of sulphite oxidation was corrected for the spontaneous reaction and the figures quoted for thiosulphate refer to the first and second phases of sulphate production.

Sulphur compound	Rate of sulphate production (μ mole/hr./ml.)
Thiosulphate	6.2
	1.1
Sulphide	1.0
Sulphur (exogenous)	0.4
(endogenous)	1.4
Sulphite	5.4

sulphur showed that endogenous sulphur accounted for up to 27 % of the dry weight of the organisms.

Sulphide, exogenous sulphur and sulphite were oxidized to sulphate by the suspensions (Table 3). Sulphur accumulated intracellularly during the oxidation of sulphide but not of sulphite. The suspensions did not oxidize tetrathionate to sulphate under anaerobic conditions in the light.

Oxidation of ^{35}S -thiosulphates

The kinetics of thiosulphate utilization and of sulphate production suggested that the accumulation of intracellular sulphur represented an intermediate stage in the oxidation of thiosulphate to sulphate by the suspensions. This possibility was examined with ^{35}S -thiosulphate labelled in the inner (SO_3^-) and outer (S-) atoms. The magnitude of intracellular sulphur accumulation and the origin of this sulphur was also determined by such isotopic techniques. Radioactivity was measured in the sulphate produced and in the organisms of the suspensions (as described in the Methods section).

Fig. 3. Labelling of organisms and sulphate in suspensions oxidizing ^{35}S -thiosulphates. Similar suspensions of thiosulphate-grown organisms containing 0.25 mg. protein/ml. were incubated under anaerobic conditions at a light intensity of 500 ft.c. in the presence of (a) inner (SO_3^-) and (b) outer (S-) labelled thiosulphate (4 mM; $0.1 \mu\text{c.}/\mu\text{mole}$). The amounts of radioactivity in the sulphate (—○—) fraction and in the organisms (—■—) were determined. The arrow indicates the completion of thiosulphate utilization.

Oxidation of $\text{S-}^{35}\text{SO}_3^{2-}$. The radioactivity of the thiosulphate was rapidly transferred to the sulphate fraction, during thiosulphate disappearance; on the completion of thiosulphate utilization, more than 90 % of the ^{35}S added originally as thiosulphate was in the sulphate fraction (Fig. 3a). There was negligible accumulation of radioactivity in the organisms.

Oxidation of $\text{S-}^{35}\text{SO}_3^{2-}$. During thiosulphate utilization, the organisms were labelled rapidly; on the completion of thiosulphate disappearance this labelling had reached a peak value equivalent to 80 % of the radioactivity added initially as thiosulphate (Fig. 3b). As the incubation continued, ^{35}S accumulated in the sulphate fraction at the expense of the radioactivity within the organisms.

The labelling of the organisms during the oxidation of outer-labelled thiosulphate coincided with the change in turbidity of the suspension. Turbidity changes have been used in this work as a rough measure of intracellular sulphur accumulation during thiosulphate oxidation by suspensions (Fig. 1) in place of the lengthy analytical methods used by other workers (van Niel, 1936; Trüper & Schlegel, 1964).

The thiosulphate-cleaving enzyme (rhodanese)

Crude extracts of the organism catalysed a cyanide-dependent cleavage of thio-sulphate to thiocyanate and sulphite, an activity attributed to the enzyme rhodanese. This reaction did not occur with heated extracts.

Rhodanese assay. An assay for the enzyme based on the spontaneous reduction of DCIP by sulphite in the presence of PMS was developed and used to investigate the properties of the enzyme in crude extracts and to follow activity during enzyme fractionation:

With partially purified pig kidney rhodanese (Westley & Green, 1959), this assay method was compared with the standard assay for the mammalian enzyme. The reaction mixture used in the conventional enzyme assay (Sorbo, 1963) was supplemented with DCIP (0.5 $\mu\text{mole}/3 \text{ ml.}$) and PMS (0.25 mg./3 ml.) and dye reduction was followed spectrophotometrically. Enzyme activity using the dye reduction method was 25% lower than that given by the standard method, but the dye reduction system had the advantage of greater rapidity and sensitivity.

Table 4. *Rhodanese activity in extracts of photosynthetic bacteria*

Extracts were prepared from organisms harvested towards the end of growth. The activity of the enzyme in extracts was determined using samples containing 0.25 mg. protein/ml. and the values were corrected for heat-stable activity.

Organism	Growth substrate	Enzyme activity (units/mg. of protein)
Chromatium D	$\text{S}_2\text{O}_3^{2-} + \text{CO}_2$	9
Chromatium D	Pyruvate	8
Chromatium D	Succinate	7
<i>Rhodospirillum rubrum</i>	Malate + glutamate + 0.2 % yeast extract	5
<i>Rhodopseudomonas spheroides</i>	Malate + glutamate	5.5

Properties of the rhodanese activity of crude extracts. The rhodanese activity of crude extracts of the organism was optimal at pH 8.7 and the Michaelis constants of the enzyme for thiosulphate and cyanide were 0.6 and 20 mM respectively. The enzyme was insensitive to EDTA (10 mM), fluoride (10 mM), azide (10 mM), thiocyanate (10 mM), cadmium ions (1 mM), arsenite (1 mM) and iodoacetate (1 mM). Tetrathionate (10–40 mM) added before or after the initiation of the reaction with cyanide inhibited the enzyme-dependent reduction of DCIP.

The enzyme was found in extracts of *Chromatium D* grown on succinate and pyruvate and of anaerobically grown *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*. The amounts of enzyme in the extracts of the Athiorhodaceae were approximately half that in the extracts of inorganic grown *Chromatium D* (Table 4). The activity of the bacterial extracts was less than that in the pig kidney extract; this gave a value of 44 units/mg. protein when assayed in the same way.

Partial purification of the enzyme. The enzyme in crude extracts of inorganic grown *Chromatium D* was partially purified in a six-stage process (Table 5)

Fig. 4. Chromatography of rhodanese on DEAE cellulose. The protein fraction from stage V was applied to a column of DEAE cellulose. 15 % of the total enzyme recovered passed through the column at low ionic strength (column fractions 10–15 termed fraction A); the remainder of the enzymic activity was eluted from the column with 350–400 mM buffer (column fractions 49–57 termed fraction B). Protein (----), buffer concentration (— · — ·) and enzyme activity (—) were determined.

Table 5. *Partial purification of the rhodanese activity in crude extracts of Chromatium D*

The details of the individual steps are given in Methods. 5 ml. of the concentrated protein fraction from stage IV were applied to the column of Sephadex before chromatography on DEAE cellulose.

Stage	Treatment	Product volume (ml.)	Total protein (mg.)	Total enzyme (units)	Specific activity (units/mg. protein)
I	French press	30	1050	12000	11
II	High-speed centrifugation	25	450	8550	19
III	Streptomycin sulphate	28	386	7050	21
IV	Ammonium sulphate 0–90 % saturation	18	325	6120	19
V	Desalting with Sephadex	12	124	2107	917
VI	Chromatography on DEAE cellulose				
	Fraction A nos. (10–15)	30	0.5	148	296
	Fraction B (nos. 49–57)	45	22	887	40

involving differential centrifuging, the precipitation of nucleic acid and enzyme with streptomycin sulphate and ammonium sulphate respectively, gel filtration on Sephadex and column chromatography on substituted celluloses. The enzyme was recovered from DEAE cellulose as well as from CM cellulose in two distinct fractions (Fig. 4). The smaller fraction (A) was unstable during storage and unsuitable for further study; the enzyme in the other fraction (B) was similar in optimum pH, Michaelis constant for thiosulphate and inhibition by tetrathionate to the activity of crude extracts. The enzyme in fraction B, after dialysis, was eluted from DEAE cellulose as a single peak of activity.

DISCUSSION

The growth of *Chromatium* sp. strain D under autotrophic conditions on sulphide, thiosulphate, exogenous sulphur, and sulphite confirmed the observations of van Niel (1931). The ability to grow on thiosulphate is a feature which *Chromatium* D has in common with the small Thiorhodaceae (thiopedia and thiocapsa species) and *Chlorobium thiosulphatophilum* and which distinguishes it from the large purple sulphur bacteria (*Chromatium okenii*, *C. warmingii*, *Thiospirillum jenense*) and *Chlorobium limicola* (see Trüper & Schlegel, 1964). Tetrathionate, a suggested intermediate in the microbial oxidation of thiosulphate, did not support the growth of *Chromatium* D under autotrophic conditions.

Various workers have reported the intracellular accumulation of sulphur during the growth of Thiorhodaceae on sulphide and thiosulphate (van Niel, 1931, 1936; Eymers & Wassink, 1937). Sulphur accumulation was a characteristic feature of the growth of *Chromatium* D on sulphide and thiosulphate and of the oxidation of these sulphur compounds by suspensions of the organism. This sulphur is an endogenous counterpart of the exogenous sulphur compounds required for autotrophic growth as it supported the further growth of the organism in a sulphur-free medium. Endogenous sulphur accounted for up to 27 % of the dry weight of the organism; similar amounts of sulphur accumulate in other Thiorhodaceae during growth on sulphide (van Niel, 1936; Trüper & Schlegel, 1964). The chemical nature of this endogenous sulphur is not known. Intracellular sulphur bears some analogy to poly- β -hydroxybutyrate, which accumulates in photosynthetic bacteria during growth under certain conditions on organic media (Stanier, Doudoroff, Kunisawa & Contopoulou, 1959; Doudoroff & Stanier, 1959). Endogenous sulphur resembles the polymer in the magnitude of accumulation, in its requirements for utilization and in its ability to support further growth. Both accumulants are a potential source of reducing power within the organism although poly- β -hydroxybutyrate is also a source of fixed carbon.

During thiosulphate utilization by suspensions of *Chromatium* D, the inner (SO_3^-) atom was rapidly oxidized to sulphate whilst the outer (S-) atom largely accumulated as endogenous sulphur. On the completion of thiosulphate disappearance, more than 90 % of the inner atom and 15 % of the outer atom of thiosulphate were in the sulphate fraction; the remainder of the outer atom was within the organisms and was oxidized to sulphate at a relatively slow rate compared with the inner atom. At least 80 % of the outer atom of thiosulphate passed through the stage of endogenous sulphur, implying that it was an intermediate in the oxidation

of thiosulphate to sulphate by Chromatium D. Endogenous sulphur has a similar intermediate role in the oxidation of sulphide by *Chromatium okenii* (Trüper, 1964). In the present work the results indicated that the inner and outer sulphur atoms of thiosulphate were oxidized at different rates and therefore independently to sulphate. This requires the separation of the two sulphur atoms of thiosulphate at an early stage in the oxidation process. The fact that Chromatium D grew on, and oxidized, sulphide, sulphur and sulphite but not tetrathionate was consistent with the key role envisaged for thiosulphate cleavage in the oxidation process (Fig. 5). The role of rhodanese in the oxidation of thiosulphate by Chromatium D remains to be proved, especially since the enzyme was also found in Athiorhodaceae. It could, however, be concerned in the initial cleavage reaction. A double displacement mechanism has recently been proposed for the reaction catalysed by the mammalian enzyme (Green & Westley, 1961; Westley & Nakamoto, 1962; Villarejo & Westley, 1963*a, b*) involving the formation of an enzyme-sulphur intermediate.

Fig. 5. The complete oxidation of thiosulphate.

The enzyme is regenerated in a reaction with an acceptor molecule such as cyanide or reduced lipoic acid. In the case of the oxidation of thiosulphate by Chromatium D, a reaction mechanism is envisaged in which a physiological acceptor reacts with an enzyme-sulphur intermediate to give sulphur or a closely related compound. Reactions other than the cyanide-dependent cleavage of thiosulphate catalysed by Chromatium rhodanese have yet to be demonstrated.

Although the mechanism of the oxidation of inorganic sulphur compounds by coloured and colourless sulphur bacteria are apparently different in detail, the oxidation process in both groups of organisms is closely associated with the production of reducing power for the fixation of carbon dioxide and subsequent biosynthetic reactions. The growth yields of Chromatium D in media containing a variety of reduced inorganic sulphur compounds were consistent with the electron donor role of the sulphur compound in bacterial photosynthesis (van Niel, 1931). The nature of the electron acceptors directly involved in the oxidation of sulphur compounds by Chromatium D is, however, unknown.

This research was aided by a grant from the Department of Scientific and Industrial Research and from the Rockefeller Foundation. A.J.S. wishes to thank the Agricultural Research Council for a Research Studentship.

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