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Control of 5-aminolaevulinate Synthetase Activity in Rhodopseudomonas spheroides [and Discussion]

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Control of 5-aminolaevulinate synthetase activity in *Rhodopseudomonas spheroides*

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Rhodopseudomonas spheroides can grow in a defined medium with either light or oxygen as an energy source. Cells grown anaerobically or at very low oxygen tensions are rich in the photosynthetic pigment bacteriochlorophyll, whereas this pigment is virtually absent in cells grown under high oxygen tensions. Aminolaevulinate synthetase, the first enzyme on the pathway to bacteriochlorophyll, appears to play an important role in the control of bacteriochlorophyll synthesis. Thus, the enzyme has a high activity in extracts of pigmented cells and a low activity in extracts of non-pigmented cells. Further, oxygenation of a pigmented culture causes immediate cessation of pigment synthesis and produces a rapid fall in the activity of aminolaevulinate synthetase. This loss of activity appears to be due to the loss of an endogenous activator of the enzyme. Thus, pigmented cells contain cystine trisulphide, which at μM concentrations is an activator of aminolaevulinate synthetase, while oxygenation causes a rapid fall in the cellular content of this trisulphide. Cystathionase (EC 4.2.1.15) extracted from pigmented cells can catalyse the formation of cystine trisulphide from cystine, while rhodanese (EC 2.8.1.1) extracted from the same cells can catalyse the degradation of cystine trisulphide in the presence of sulphite to form cystine and thiosulphate.

It is proposed that the cellular content of cystine trisulphide is controlled by changes in the levels of substrates for cystathionase and possibly rhodanese rather than changes in the amounts of these enzymes. Cystine trisulphide controls the activity of aminolaevulinate synthetase by converting a low-activity form of the enzyme (b-form) into a high-activity form (a-form). The fall in aminolaevulinate synthetase activity on oxygenation appears to be the result of cessation of conversion of b-form into a-form, along with a conversion of a-form into b-form. Factors affecting the equilibrium between the forms and the possible mechanisms for their interconversion are discussed.

Introduction

The non-sulphur purple bacterium *Rhodopseudomonas spheroides*, a member of the group Athiorhodaceae, can grow in a defined medium with either light or oxygen as an energy source. This organism has a branched pathway of tetrapyrrole biosynthesis producing the three functional tetrapyrrole derivatives bacteriochlorophyll, haem and vitamin B_{12} (figure 1). In *R. spheroides* and related organisms marked variations in the cellular content of these products occur in response to environmental changes. Cells grown under high oxygen tensions have a respiratory type of metabolism and are virtually devoid of the photosynthetic pigment bacteriochlorophyll. When these cells are transferred to conditions of low oxygen tension there follows a process of adaptation resulting in the synthesis of large amounts of bacteriochlorophyll. When these now pigmented cells, growing under photosynthetic conditions, are suddenly exposed to a high oxygen tension, pigment synthesis ceases immediately, while growth proceeds normally. These changes in the bacteriochlorophyll content of cells are accompanied by similar, but far less dramatic changes in the content of haem and vitamin B_{12}. The mechanism by which oxygen tension and light intensity control this pathway has been a subject for investigation in this

and several other laboratories since the problem was first described in detail (Cohen-Bazire, Sistrom & Stanier 1957).

Lascelles (1959, 1960) found that the activity of aminolaevulinate synthetase (see figure 1) in cells grown under different conditions could be directly correlated with the rate of synthesis of bacteriochlorophyll; it was suggested that oxygen might control the pathway by altering the rate of synthesis of aminolaevulinate synthetase and possibly other control enzymes. It was further proposed that, since oxygenation of a pigmented culture causes immediate cessation of bacteriochlorophyll synthesis, oxygen must produce a direct inhibition of one or more enzymes of the pathway, in addition to its effect on their rate of synthesis.

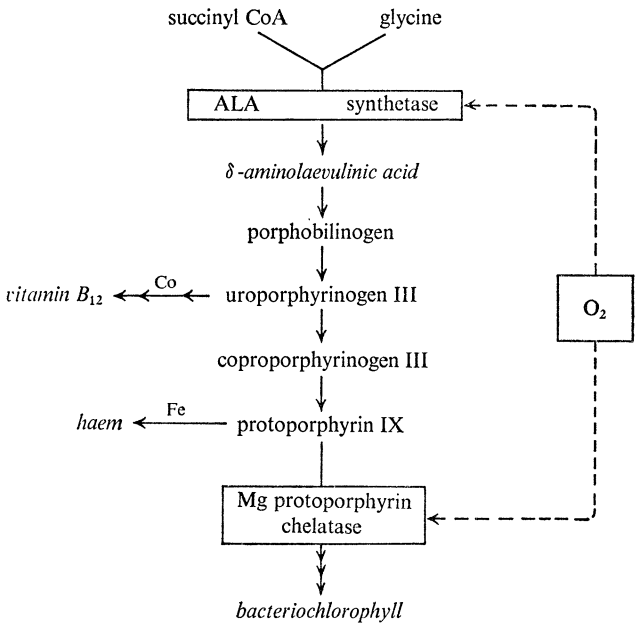

FIGURE 1. Tetrapyrrole biosynthesis in *R. spheroides*.

Oxygen is now considered to inhibit bacteriochlorophyll synthesis directly at two or more points on the pathway (see figure 1). Work with a mutant of *R. spheroides* (see Lascelles & Altshuler 1969) which lacked aminolaevulinate synthetase and which therefore required exogenous aminolaevulinate for growth indicated that oxygen has a direct inhibitory effect on magnesium protoporphyrin chelatase. In this mutant oxygen inhibited the synthesis of magnesium derivatives of protoporphyrin but not the synthesis of haem. Indeed, inhibition of magnesium-insertion by high oxygen tension was later directly demonstrated by assay of the enzyme in whole cell suspensions (Gorchein 1972). It is clear, however, that oxygen must produce a simultaneous fall in the production of aminolaevulinate since inhibition by oxygen of magnesium protoporphyrin chelatase, does not result in the accumulation of aminolaevulinate, porphobilinogen or other precursors of protoporphyrin. That this fall is due to a markedly diminished activity of aminolaevulinate synthetase was shown by direct assay of the enzyme in cell extracts (cf. Marriott, Neuberger & Tait 1969).

Lascelles (1968) has explained this non-appearance of precursors by a control mechanism in which the primary effect of oxygen is inhibition of magnesium insertion. This results in the diversion of protoporphyrin from the synthesis of bacteriochlorophyll into that of haem which

acts as a feed-back inhibitor of aminolaevulinate synthetase. It had been shown earlier that haem is a potent inhibitor of aminolaevulinate synthetase activity *in vitro* (Gibson, Matthews, Neuberger & Tait 1961). This attractive hypothesis has not been tested directly since it is not possible to measure the cellular content of free haem. In addition cells are impermeable to haem added to the medium. Further, Lascelles has commented that regulation of aminolaevulinate synthetase in *R. spheroides* by haem alone seems paradoxical in that quantitatively the main function of the enzyme is in bacteriochlorophyll synthesis.

A different control mechanism had been proposed earlier by Cohen-Bazire *et al.* (1957). It was suggested that pigment synthesis is controlled by the state of oxidation of a carrier in the electron transport system, and that oxygenation of a growing culture oxidizes the regulatory carrier thus leading to inhibition of pigment synthesis. The structure of the regulator and the nature of its effect on the pathway remain unknown. Some evidence for the participation of the electron transport system in the control of bacteriochlorophyll synthesis has been given (Davies *et al.* 1973). Thus, electron transport between a *b*-type and a *c*-type cytochrome appears to be necessary for the enzymic insertion of magnesium into protoporphyrin. Also, the spontaneous activation of aminolaevulinate synthetase in cell-free extracts of *R. spheroides* occurs only in the presence of active electron transport (Marriott, Neuberger & Tait 1970). Marrs & Gest (1973), however, have provided evidence that inhibition of bacteriochlorophyll synthesis by oxygen cannot be entirely explained by alterations in the redox state of the electron transport chain. Thus, bacteriochlorophyll synthesis in mutants of *R. capsulata*, which are deficient in various activities of the respiratory electron transport system, is inhibited by oxygen to the same extent as in the wild-type. They have proposed that oxygen directly inactivates an unknown factor which is essential to bacteriochlorophyll synthesis, and that reactivation of this factor, is mediated by electron flow from the electron transport system.

The idea of an oxygen-sensitive factor is consistent with our recent proposal that oxygen controls aminolaevulinate synthetase activity in *R. spheroides* by altering the cellular content of an activator of the enzyme (Davies *et al.* 1973). *R. spheroides* growing semi-anaerobically in the light contains cystine trisulphide which at μM concentrations is an activator of aminolaevulinate synthetase. Oxygenation of a culture produces a marked disturbance of the sulphur metabolism of the cell which results in gross depletion of GSH + GSSG, cysteine + cystine (Neuberger, Sandy & Tait 1973*a*) and a virtual disappearance of cystine trisulphide (Sandy, Davies & Neuberger 1975).

This proposed control of aminolaevulinate synthetase activity by sulphur compounds of low molecular mass appears to be related to the occurrence of multiple forms of the enzyme described by Tuboi and his co-workers. Aminolaevulinate synthetase has been resolved into low activity enzyme (b-forms) and high activity enzyme (a-forms) by chromatography on DEAE-Sephadex (Tuboi & Hayasaka 1973). Activation of the enzyme (b-form to a-form) could be achieved by the addition of any of a variety of disulphides and an endogenous activating enzyme, while inactivation (a-form to b-form) was obtained on treatment with thiol compounds (cysteine, mercaptoethanol, dithiothreitol) and an endogenous insoluble factor. Interconversion of the forms does not involve significant alterations in the apparent molecular mass of the enzyme but alters the chromatographic properties on DEAE-Sephadex. Fanica-Gaignier & Clément-Métral (1973) have also isolated two forms of the enzyme from *R. spheroides* strain Y and found that they differ in their isoelectric points and by the number of titratable –SH groups.

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We have also observed multiple forms of aminolaevulinate synthetase on fractionation of extracts on DEAE-Sephadex. Our low activity enzymes (b-forms) can be activated two- to fivefold by cystine trisulphide (1–5 μM), while the high activity enzyme (a-form) is unaffected (Sandy *et al.* 1975). A freshly prepared extract of cells grown semi-anaerobically in the light contains a mixture of a-form and b-form enzyme. On storage of this extract at 4 °C there is a conversion of b-form to a-form which explains the spontaneous activation of the enzyme observed by Marriott *et al.* (1969). Extracts of cells which were grown semi-anaerobically but oxygenated before being harvested, contain exclusively b-form enzyme. When the enzyme in these extracts was activated by cystine trisulphide only a-form enzyme was recovered, indicating that trisulphide had converted the b-form into the a-form.

The possible involvement of trisulphides in the control of aminolaevulinate synthetase has prompted us to investigate the modes of synthesis and degradation of cystine trisulphide in *R. spheroides*. A role for cystine trisulphide, and possibly for glutathione trisulphide, in transulphuration reactions was suggested by Szczepkowski & Wood (1967). They found that cystathionase (EC 4.2.1.15) purified from rat liver could form cystine trisulphide from cystine, and that the labile sulphur of the trisulphide could then be transferred to cyanide in the presence of rhodanese (EC 2.8.1.1). They suggested that trisulphides may act as intermediates in transulphuration from cystine to an acceptor molecule, although there is no independent evidence for the occurrence of this pathway. The mechanism by which cystine trisulphide is formed in the cystathionase dependent cleavage of cystine is not fully understood. The reaction was first studied by Cavallini, DeMarco, Mondovi & Mori (1960*a*), who suggested that the primary sulphur-containing product would be thiocysteine (cysteine persulphide) (reaction (1)):

$$\mathrm{CySSCy} + \mathrm{H_2O} \xrightarrow{\text{cystathionase}} \mathrm{CyS\text{-}SH} + \text{pyruvate} + \mathrm{NH_3}. \qquad (1)$$

It has not been possible to isolate free thiocysteine from incubation mixtures or to prepare the compound chemically due to its marked instability in solution (Cavallini *et al.* 1960*a*). However, Flavin (1962) was able to demonstrate the formation of *S*-carboxymethylthiocysteine in incubation mixtures containing cystathionase, cystine and iodoacetate.

The subsequent formation of cystine trisulphide in incubations with cystathionase was proposed by Szczepkowski & Wood (1967) to follow from the reaction of the unstable thiocysteine with excess cystine (reaction (2)):

$$\mathrm{CySSH} + \mathrm{CySSCy} \longrightarrow \mathrm{CySSSCy} + \mathrm{CySH}. \qquad (2)$$

The general sulphane–sulphur transferase activity of rhodanese (Westley 1973) suggested to us that this enzyme may be involved in the metabolism of trisulphides in *R. spheroides*. Rhodanese activity was measured in extracts of these organisms by Smith & Lascelles (1966) but the enzyme was not studied in detail. Active rhodanese, crystallized from ox liver is a sulphur-substituted protein, which has been shown to use cystine trisulphide as a donor substrate with either cyanide or sulphite (reaction (3)) as acceptor substrates (Szczepkowski & Wood 1967):

$$\mathrm{CySSSCy} + \mathrm{SO_3^{2-}} \longrightarrow \mathrm{CySSCy} + \mathrm{S_2O_3^{2-}}. \qquad (3)$$

Rhodanese may therefore act in the degradation of trisulphides *in vivo*. If reaction (2) is general for persulphides then it may be expected that any enzymically synthesized persulphide may convert a disulphide to a trisulphide. Such a persulphide may be formed not only by cysta-

thionase but also by rhodanese. Thus, it has been suggested (Villarejo & Westley 1963; Koj 1968) that the reaction of various thiols (cysteine, GSH, dihydrolipoate) with thiosulphate, in the presence of rhodanese, leads to the formation of a persulphide (reaction (4)):

$$\text{R-SH} + \text{S}_2\text{O}_3^{2-} \longrightarrow \text{R-S-SH} + \text{SO}_3^{2-}. \quad (4)$$

Rhodanese may therefore be involved in the synthesis of trisulphides as well as in their degradation.

In the present work with *R. spheroides* we have purified both cystathionase and rhodanese about 100-fold. Cystathionase has been found to catalyse the synthesis of cystine trisulphide from cystine. Rhodanese has been shown to catalyse not only the breakdown of cystine trisulphide but also the conversion of thiosulphate to sulphite in the presence of thiol compounds. We have also studied the effect of growth conditions on the activity of both enzymes and we have investigated the effect of specific inhibitors of cystathionase on tetrapyrrole biosynthesis in *R. spheroides*. A likely role for these enzymes in the control of aminolaevulinate synthetase in this organism is discussed.

Materials and methods

Growth and harvesting of organisms and preparation of cell-free extracts

Rhodopseudomonas spheroides (N.C.I.B. 8253) was used throughout and was maintained in stab culture as described by Lascelles (1956). The growth of organisms semi-anaerobically in the light and under oxygen in the dark, the adaptation of organisms to low oxygen tensions, the harvesting of organisms and the preparation of cell-free extracts have been described previously (Gorchein, Neuberger & Tait 1968; Neuberger *et al.* 1973*a*).

Determinations

The dry mass of organisms and the protein content of cell-free extracts was determined as described by Marriott *et al.* (1969). Bacteriochlorophyll was measured by the method of Cohen-Bazire *et al.* (1957). Radioactivity was measured on the Packard Liquid Scintillation Spectrometer (Model 544).

Other methods

The activator activity of extracts was determined as described by Neuberger, Sandy & Tait (1973*b*) except that the enzyme preparation used was the upper supernatant from step 2 of the purification procedure.

Thin-layer chromatography was done on pre-coated cellulose plates (10 cm × 20 cm) obtained from E. Merck, Darmstadt, Germany.

High-voltage electrophoresis at pH 2.0 was performed as previously described (Neuberger *et al.* 1973*a*). Electrophoresis at pH 4.3 was in pyridine acetate buffer for 90 min at 40 V/cm as described by Flavin (1962).

Radioactive compounds were located on electrophoretograms by contact with 'Kodirex' X-ray film (Kodak, London, U.K.) in a lead-backed exposure holder for between 1 and 3 days.

Homocysteine and cysteine in cystathionase incubations were determined as homocysteic acid and cysteic acid after performic acid oxidation of protein-free extracts. The two acids were

distinguished by high-voltage electrophoresis at pH 2.0 and by t.l.c. in methanol:pyridine:H_2O (20:1:5, by vol.). A semiquantitative determination of the compounds was made by the intensity of the ninhydrin colour on electrophoretograms as described by Neuberger *et al.* (1973*a*).

Determination of cyanide-labile sulphur

The colorimetric procedure of Fletcher & Robson (1963) was used when sufficient material was available. A radioactivity assay was developed for determinations with 10-100 nmol of ^{35}S-labelled polysulphides. The method is based on the formation of [^{35}S]thiocyanate by alkaline cyanolysis of polysulphides. To a dried sample of radioactive polysulphide (at least 200 (count/min)/nmol of sulphur) was added HCl (25 μmol), NH_4OH (100 μmol), potassium cyanide (12.5 μmol) and water to a total volume of 1.1 ml. After incubation at 25 °C for 45 min, HCl (100 μmol) and 0.35 ml of a 50 % (by vol.) suspension of Dowex 50 (H^+ form) in water were added. The suspension was mixed, centrifuged briefly and the supernatant was retained. The pellet was washed with 1 ml of water and the total radioactivity in supernatant plus water wash was determined. Controls were done in which cyanide was omitted from the reaction but was added after the final acidification. The amount of cyanide-labile sulphur (as thiocyanate) was calculated from the known specific radioactivity of the treated compound.

Assay of enzyme activities

Aminolaevulinate synthetase activity was determined as described by Neuberger *et al.* (1973*a*).

Cystathionase

Enzyme activity was determined by three different methods.

Method 1 is based on that described by Flavin (1962) in which thiol, generated by cleavage of thioether or disulphide, is determined by reaction with DTNB (5,5′-dithiobis-[2-nitrobenzoic acid]. The incubation mixture contained substrate (2 μmol), pyridoxal phosphate (0.1 μmol), phosphate buffer pH 7.3 (100 μmol), DTNB (0.2 μmol), enzyme (0.2–3.0 mg of protein) and water to a total volume of 1.0 ml. Incubation was at 37 °C (30–90 min) and the extinction at 412 nm was measured. All assays had two control tubes, one without substrate and the other without enzyme. Both control values were substracted from the extinction obtained with the complete incubation mixture. The amount of product formed was obtained from a calibration curve obtained by the addition of cysteine (0–100 nmol) to incubation mixtures that were without substrate and enzyme. The same results were obtained when calculations were based on a molar extinction coefficient for reduced DTNB of 13 600 (Ellman 1959).

Method 2 is based on that described by Flavin (1962) in which keto-acid is determined by oxidation of NADH in the presence of lactic dehydrogenase. The incubation mixture was the same as described for method 1, but omitting the DTNB and including phosphate buffer pH 7.8 (100 μmol) instead of pH 7.3 buffer. Incubation was at 37 °C, the reaction was stopped by the addition of 0.15 ml of 25 g/100 ml trichloroacetic acid and tubes were immediately cooled to 0 °C. A portion of the protein-free supernatant was analysed for pyruvic acid and ketobutyric acid by the method described by Flavin & Slaughter (1964). All assays had two control tubes, one without substrate and the other without enzyme. Both control values were subtracted from the result obtained with the complete incubation mixture. The amount of product was obtained from a calibration curve constructed from the analysis of mixtures of known amounts of pyruvic acid and α-ketobutyric acid.

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Method 3 is based on the determination of ammonia. The incubation mixture was that described in method 1 but without DTNB. After incubation at 37 °C the ammonia was removed from solution by microdiffusion in small Conway vessels. Samples (0.4 ml) were treated with sodium carbonate (0.3 ml), the ammonia was collected over 20 h at 4 °C in 0.1 M-HCl (0.1 ml) and determined by the method of Chaney & Marbach (1962).

One unit of cystathionase is defined as the amount catalysing the formation of 1 nmol of product (thiol, keto-acid or ammonia) in 60 min at 37 °C. Determination by methods 2 and 3 gave essentially the same results, whereas method 1 gave 40–50 % lower values. This was explained by inhibition of cystathionase activity by DTNB. Thus, 0.2 mM DTNB inhibited the formation of ammonia by about 50 % in incubation of cystathionase with DL-cystathionine as substrate (cf. Guggenheim 1970).

Rhodanese

Enzyme activity was determined by two different methods.

Method 4 is described by Smith & Lascelles (1966). Sulphite, generated from thiosulphate (50 mM), is determined by reduction of the dye dichlorophenolindophenol (DCPIP). One unit of enzyme is defined as the amount catalysing the reduction of 1 μmol of DCPIP per minute.

Method 5. The incubation mixture was based on that of Smith & Lascelles (1966) with one-third the amount of all constituents, and without DCPIP and phenazine methosulphate. Incubation was at 25 °C and the reaction was stopped by the addition of 0.2 ml formaldehyde solution (38 %, by vol., in water) and 1.0 ml of ferric nitrate reagent (Sorbo 1953). After centrifugation, the extinction at 460 nm was measured. The amount of thiocyanate formed was calculated from a standard curve obtained with potassium thiocyanate. One unit of enzyme is defined as the amount catalysing the formation of 1 μmol of thiocyanate per minute.

Determination of other products formed in rhodanese incubations

Sulphite determination

In experiments with thiol compounds as substrates the DCPIP method of sulphite determination (method 4 above) could not be used due to reduction of the dye by substrate. Under these conditions sulphite was determined by a method based on that described by Sorbo (1958). The rhodanese reaction was stopped by the addition of an equal volume of 0.2 M $HgCl_2$. To 0.5 ml of the protein-free supernatant were added 2 ml of 0.04 g/100 ml *p*-rosaniline in 0.72 M HCl and 2 ml of formaldehyde (0.2 %, by vol., in water). The extinction at 570 nm was measured and the amount of sulphite was calculated from a calibration curve made with sodium sulphite.

Thiosulphate determination

This is an adaptation of the method described by Sorbo (1957). The pH of an aliquot of the incubation mixture was adjusted to 10 with 3 M NaOH, and water was added to a total volume of 4.2 ml. Sodium cyanide (0.5 ml, 0.1 M) was added, the sample was mixed and $CuCl_2$ (0.3 ml, 0.1 M) was added with rapid mixing. After the addition of 0.5 ml of ferric nitrate reagent (Sorbo 1953) the solution was centrifuged and the extinction at 460 nm was measured. The amount of thiosulphate was calculated from a standard curve constructed with known amounts of sodium thiosulphate taken through the procedure.

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Chemicals

DTNB, *p*-rosaniline hydrochloride, phenazine methosulphate, DL-2,3-diaminopropionic acid, L- and DL-cystathionine, pyruvic acid, α-ketobutyric acid, β-cyanoalanine and reduced glutathione were obtained from Sigma (London) Chemical Co. Ltd, London, S.W. 6, U.K.; *S*-carboxymethyl-L-cysteine, *S*-ethylamino-L-cysteine, DL-lanthionine, L-cysteine, DL-serine, DL-homoserine, sodium thiosulphate (A.R.), sodium sulphite (A.R.), sodium thiocyanate (A.R.), sodium cyanide (A.R.) and pyridoxal phosphate from British Drug Houses Chemicals Ltd, Poole, Dorset, U.K. *S*-Sulpho-L-cysteine was prepared as described by Segal & Johnson (1963), cystine trisulphide as described by Fletcher & Robson (1963) and *S*-carboxymethyl-thiocysteine as described by Flavin (1962). Lactate dehydrogenase and NADH were from Boehringer Corp. (London) Ltd, London, W. 5, U.K. L-Homolanthionine was a gift from Dr H. T. Huang, International Minerals and Chemical Corp., Libertyville, Ill., U.S.A. *S*-Methyl-L-cysteine and *S*-methyl-L-cysteine sulphoxide were a gift from Dr Smith, Rowett Institute, Bucksburn, Aberdeenshire, U.K.; [^{35}S]cystine was from the Radiochemical Centre, Amersham, Bucks, U.K. Phosphate buffers were prepared from KH_2PO_4 and KOH. Other chemicals were obtained from the sources described previously (Neuberger *et al.* 1973*a*).

Purification of enzymes

Cystathionase

All procedures were carried out at 0–4 °C. *R. spheroides* grown semi-anaerobically in the light (10 g dry mass, 100 ml) were disrupted by sonication and centrifuged at 25000 *g* for 20 min. To the supernatant (crude extract) was added 240 ml of 0.05 M phosphate buffer pH 7.3 and the whole was centrifuged at 120000 *g* for 2 h. The supernatant (330 ml) was treated with ammonium sulphate and the material which precipitated between 30 and 50 % saturation was collected. The precipitate was dissolved in 0.01 M phosphate buffer pH 7.3 (16 ml), dialysed overnight against the same buffer and applied to a column (1.6 cm × 20 cm) of DEAE cellulose previously equilibrated with this buffer. The column was washed with 125 ml of 0.1 M phosphate buffer pH 7.3 and the enzyme was eluted with a linear gradient made from 250 ml each of 0.1 M phosphate buffer pH 7.3 and 0.7 M phosphate buffer pH 7.3. Fractions with enzyme activity were combined and ammonium sulphate was added to 80 % saturation. The precipitate was dissolved in 0.05 M phosphate buffer pH 7.3 (8 ml), applied to a column of Sephadex G-200 (3.2 cm × 50 cm) and the column was eluted with the same buffer. Fractions with enzyme activity were combined and ammonium sulphate was added to 80 % saturation. The precipitate was dissolved in 0.05 M tris-HCl buffer pH 7.4, dialysed for 6 h against the same buffer and applied to a column (1.6 cm × 20 cm) of DEAE Sephadex A-25, previously equilibrated with 0.05 M tris-HCl, pH 7.4 (see figure 2). Elution was performed as decribed in step 4 of the purification of the FIb activating enzyme of aminolaevulinate synthetase (Tuboi & Hayasaka 1972). Fractions with high enzyme activity were combined and stored at −20 °C. Relevant data are summarized in table 1. Enzyme recovered from the DEAE Sephadex step was used in the studies on the synthesis of cystine trisulphide. Enzyme recovered from the Sephadex G-200 step was used in all other work; it retained 75–80 % of its activity after two months' storage at −20 °C. The apparent molecular mass of cystathionase as determined by gel-filtration on Sephadex G-200 was about 55000.

Rhodanese

The early steps of the purification of rhodanese were as described in the purification of cystathionase. The supernatant from the high-speed centrifugation (300 ml) was treated with ammonium sulphate and the material which precipitated between 40 and 65 % saturation was collected. The precipitate was dissolved in 0.05 M phosphate buffer pH 7.3 (20 ml) and purified by fractionation on the Sephadex G-200 and DEAE Sephadex columns used in the purification of cystathionase. Relevant data are summarized in table 2. Enzyme recovered from the

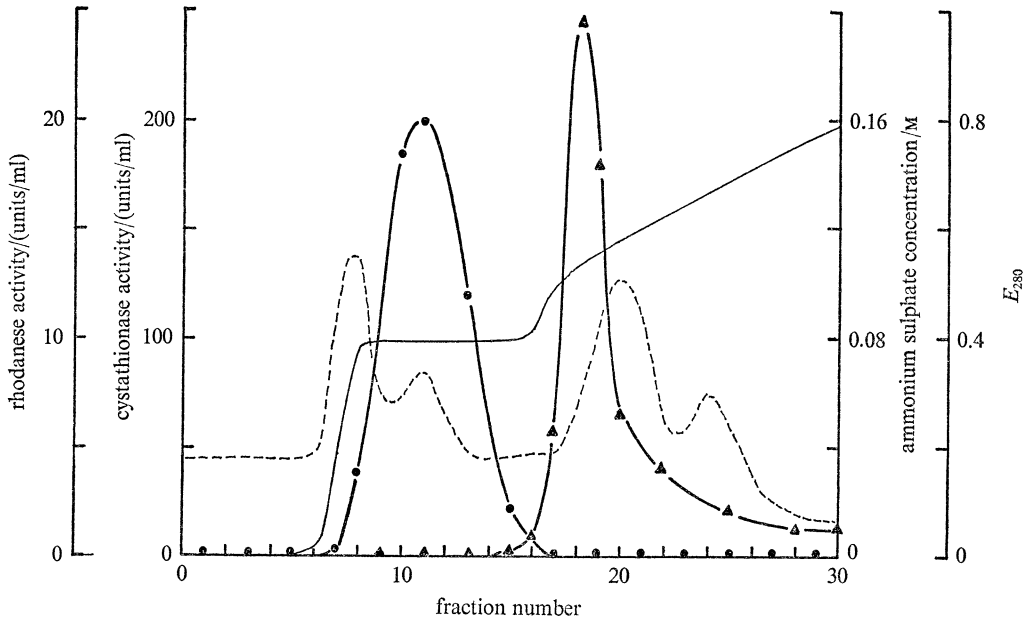

FIGURE 2. Chromatography on DEAE Sephadex of cystathionase and rhodanese from *R. spheroides*. A purified sample of cystathionase (16000 units, 34 mg of protein) was applied to a column (1.6 cm × 20.0 cm) of DEAE Sephadex A-25 previously equilibrated with 0.05 M tris/HCl buffer, pH 7.4. The column was eluted at 25 ml/h with 100 ml of 0.05 M tris/HCl, pH 7.4, containing 0.08 M ammonium sulphate, followed by a linear gradient formed from 150 ml each of 0.05 M tris/HCl buffer, pH 7.4, containing 0.08 M ammonium sulphate and 0.05 M tris/HCl buffer, pH 7.4, containing 0.2 M ammonium sulphate, and 10 ml fractions were collected. Cystathionase activity (▲) and protein concentration as estimated by E_{280} (- - - - -) were determined after dialysis of samples against 0.05 M tris/HCl buffer pH 7.4.

The elution profile for a similarly purified sample (2600 units, 105 mg of protein) of rhodanese, fractionated in a separate experiment on the DEAE Sephadex system described above, is also shown (●).

TABLE 1. PURIFICATION OF CYSTATHIONASE

fraction	volume ml	total activity unit	total protein mg	specific activity unit/mg
crude extract	95	40850	6050	6.7
high-speed supernatant	325	40600	2010	20
ammonium sulphate (30–50 % satn)	16	29700	412	72
DEAE cellulose	82	22700	145	157
Sephadex G-200	40	20700	43	485
DEAE Sephadex	25	7500	7.9	945

The activities were determined by method 2 with L-cystine as substrate. Essentially the same activities were obtained with DL-cystathionine as substrate. Units of cystathionase activity are defined in the Methods section.

DEAE Sephadex step (see figure 2) was used in all studies. These preparations retained 90 % activity after 2 months' storage at −20 °C. The apparent molecular mass of rhodanese as determined by gel-filtration on Sephadex G-200 was about 45 000.

TABLE 2. PURIFICATION OF RHODANESE

fraction	volume / ml	total activity / unit	total protein / mg	specific activity / unit/mg
crude extract	110	3200	7900	0.41
high-speed supernatant	308	3500	4240	0.83
ammonium sulphate (40–65 % satn)	20	3200	1050	3.0
Sephadex G-200	55	3050	197	15.5
DEAE Sephadex	65	1100	24	46

The activities were determined by method 4. Essentially the same activities were obtained with method 3. Units of rhodanese activity are defined in the Methods section.

RESULTS

Cystathionase-dependent synthesis of cystine trisulphide

Cystine trisulphide can be detected at μM concentrations by its activating effect on aminolaevulinate synthetase (Neuberger, Sandy & Tait 1973*c*). We obtained evidence for the enzymic synthesis of trisulphide in cystathionase incubations with cystine by showing the formation of an activator of aminolaevulinate synthetase (figure 3). The products of the incubation of cystathionase with [^{35}S]DL-cystine were identified after fractionation of the mixture on Dowex 50 (figure 4). Also shown is the elution pattern obtained in a control experiment in which denatured enzyme was used and in which no activator was formed. It is clear that compounds A, C and D were generated by the action of cystathionase on cystine. Compound B was unchanged cystine.

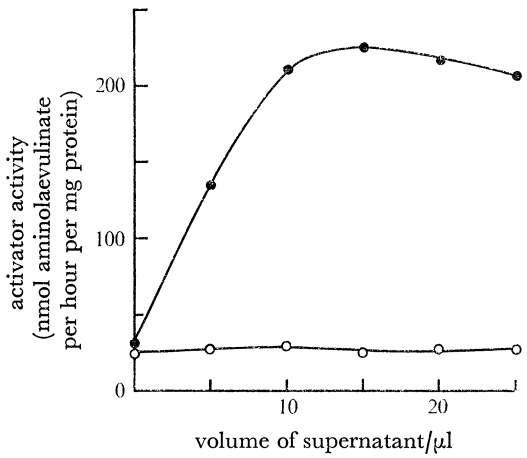

FIGURE 3. The formation of an activator of aminolaevulinate synthetase in a cystathionase incubation with cystine as substrate. The incubation contained DL-cystine (2 μmol), phosphate buffer, pH 7.5 (0.04 μmol), cystathionase (0.95 mg of protein) and water (total volume, 1.0 ml). The mixture was adjusted to pH 7.4 before adding the enzyme. Incubation was at 37 °C for 60 min and the reaction was stopped with 0.15 ml of trichloroacetic acid (15 g/100 ml). The protein-free supernatant was neutralized with KOH and aliquots were assayed immediately for activator activity (●) towards aminolaevulinate synthetase (see Methods). The assay of aliquots from a control incubation in which heat-denatured enzyme was used is also shown (○).

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Compound A exhibited no activator activity at μM concentrations and it was identified as cysteine on the basis of the following results:

(i) It co-chromatographed with L-cysteine on the Dowex 50 system described in figure 4.

(ii) When oxidized with performic acid it was converted into a compound which was identified as cysteic acid by high-voltage electrophoresis and t.l.c. as described previously (Neuberger *et al.* 1973*a*).

(iii) When treated with sodium iodoacetate at pH 7.4 it was converted into a compound which behaved identically with *S*-carboxymethyl cysteine on high-voltage electrophoresis (pH 4.3) and on t.l.c. in butanol:acetic acid:water (2:1:1, by vol.), $R_f = 0.35$.

Compound C at μM concentrations produced up to a sixfold activation of aminolaevulinate synthetase. It has been identified as cystine trisulphide on the basis of the following results:

(i) It co-chromatographed with authentic cystine trisulphide on the Dowex 50 system described in figure 4.

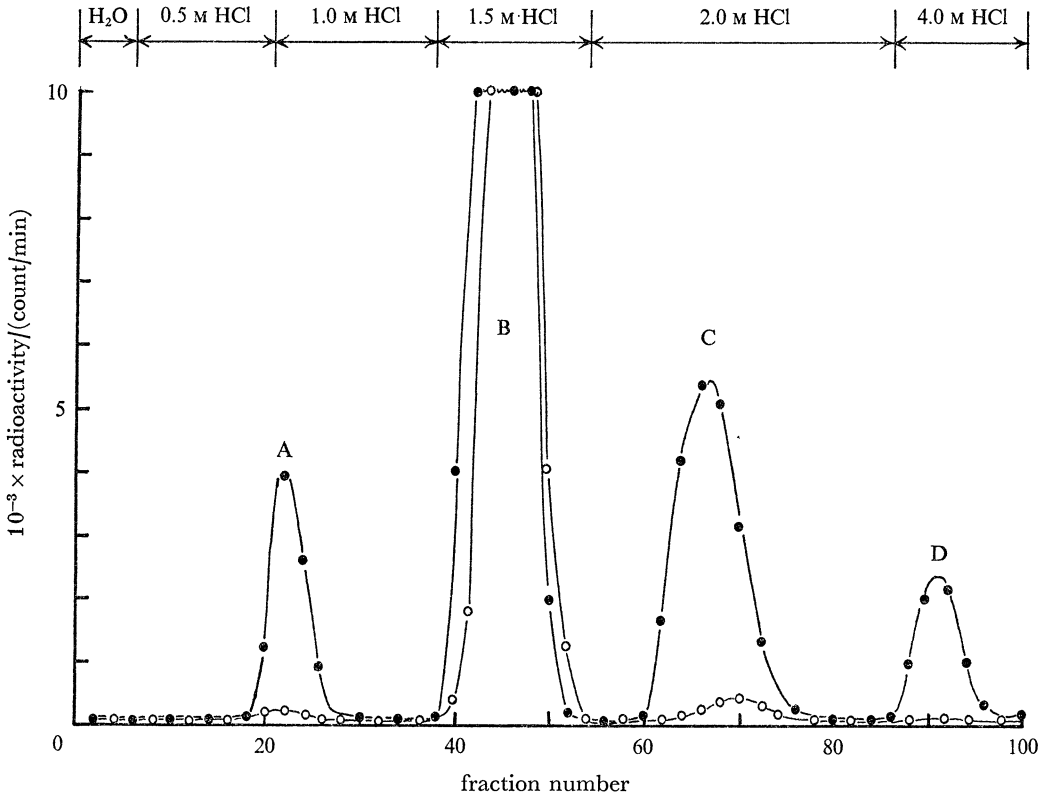

FIGURE 4. Chromatography on Dowex 50 of products of the cystathionase-dependent cleavage of cystine. The incubation contained [^{35}S]DL-cystine (2 μmol, 5.7 μCi), phosphate buffer, pH 7.5 (0.04 μmol), cystathionase (0.95 mg of protein) and water (total volume, 1.0 ml). The mixture was adjusted to pH 7.4 before adding the enzyme. Incubation was at 37 °C for 60 min and the reaction was stopped with 0.15 ml of trichloroacetic acid (15 g/100 ml). The protein-free supernatant was neutralized with KOH and 0.5 ml was applied to a column (0.9 cm × 10.0 cm) of Dowex 50 (×2; H^+ form; 200–400 mesh). The column was eluted at 36 ml/h with (i) water (10 ml), (ii) 0.5 M HCl (50 ml), (iii) 1.0 M HCl (50 ml), (iv) 1.5 M HCl (50 ml), (v) 2.0 M HCl (100 ml) and (vi) 4.0 M HCl (50 ml) and 3 ml fractions were collected. Samples were dried under vacuum in a desiccator containing KOH and aliquots (0.25 ml) were analysed for radioactivity (●). The fractionation of a control incubation mixture in which heat-denatured enzyme was used is also shown (○).

(ii) When oxidized with performic acid it was converted into a new compound which was identified as cysteic acid as described above.

(iii) It behaved identically with cystine trisulphide on high-voltage electrophoresis at pH 2.0. Mobility relative to alanine = 0.48.

(iv) Determination of the percentage of cyanide-labile sulphur gave 30.2%, 31.4% and 33.4% in three determinations. Parallel analyses of synthetic [^{35}S]cystine trisulphide (Neuberger *et al.* 1973*c*) gave values of 31.4%, 31.5% and 32.4%.

Compound D at μM concentrations produced up to a sixfold activation of aminolaevulinate synthetase. The chromatographic behaviour of this compound on Dowex 50 is consistent with it being cystine tetrasulphide (Fletcher & Robson 1963). The amount of cyanide-labile sulphur, as a percentage of the total sulphur, in compound D was 46.2%, 49.1% and 55% in three determinations while the theoretical value for cystine tetrasulphide is 50%. In addition oxidation of compound D converted it into a compound which was identified as cysteic acid as described above.

While the presence of cysteine and cystine trisulphide in incubations of cystathionase with cystine is consistent with reactions 1 and 2 shown above the small amount of cystine tetrasulphide may arise from oxidation of thiocysteine by air (reaction (5)):

$$4\mathrm{CySSH} + \mathrm{O_2} \longrightarrow 2\mathrm{CySSSSCy} + 2\mathrm{H_2O}. \qquad (5)$$

TABLE 3. PRODUCTS OF THE CYSTATHIONASE-DEPENDENT CLEAVAGE OF CYSTINE

product	amount formed (nmol)
pyruvate	340
ammonia	360
cystine trisulphide	270
cystine tetrasulphide	68
cysteine	208

The conditions of incubation were as described in the legend to figure 4. Pyruvate and ammonia were determined as described in the Methods section. The methods of isolation and determination of the sulphur compounds are described in the text.

We have found that thiocysteine is a primary product of the cleavage of cystine by the enzyme from *R. spheroides*. Thus, when cystathionase and cystine were incubated as described in the legend to figure 4, but with sodium iodoacetate (8 mM) added to trap thiol intermediates, no trisulphide or tetrasulphide was formed. The products of this reaction were fractionated on high-voltage electrophoresis (pH 4.3) and the radioactive sulphur compounds were visualized by autoradiography. The major acidic sulphur compound present was recovered from the paper and found to behave identically with authentic *S*-carboxymethylthiocysteine on high-voltage electrophoresis at pH 4.3 and on t.l.c. in butanol:acetic acid:water (2:1:1, by vol.) R_f = 0.39 (cf. Schöberl, Tausant & Gräfje 1956). The compound was also distinguished from *S*-carboxymethylcysteine by spraying the electrophoretograms with a cyanide-nitroprusside reagent (Toennies & Kölb 1951). No *S*-carboxymethylthiocysteine was detected in incubations done either with denatured enzyme or without iodoacetate.

The amounts of end-products formed in the incubation mixture described in the legend to figure 4 are shown in table 3. The sulphur compounds were isolated by chromatography on Dowex 50 and the amounts were determined by the total radioactivity. The results indicate

that about 18 % of the cystine was cleaved, producing approximately equal amounts of pyruvate and ammonia. If the reactions (1), (2), and (5) describe fully both the primary and secondary reactions occurring in the incubation then the amount of the primary product, thiocysteine, should be equivalent to that of pyruvate or ammonia. It should also be equivalent to the amount of cystine trisulphide plus twice the amount of cystine tetrasulphide. In fact this value (406 nmol) is only slightly in excess of the amounts of pyruvate or ammonia. Thus, these results seem compatible with the proposed sequence of reactions. The amount of cysteine formed is lower than the expected value and this may be due to autoxidation or possibly other side reactions.

Cystine can be cleaved non-enzymically by pyridoxal and copper ions at pH 8.5 (Cavallini, De Marco & Mondovi 1960*b*). We have found that under the conditions of our cystathionase assay (method 1) significant cleavage of cystine occurs in the absence of enzyme, but only in the presence of pyridoxal phosphate. Consequently, no pyridoxal phosphate was added to incubation mixtures in experiments designed to demonstrate the formation of cystine trisulphide. Our purified cystathionase preparation exhibited about 70 % of its full activity in assays without added pyridoxal phosphate indicating that the co-factor is firmly bound and is not resolved from the enzyme during purification. The amount of pyridoxal phosphate in our purified preparations of cystathionase was determined spectrophotometrically (Matsuo & Greenberg 1958) and found to be less than 5 nmol/mg protein. This concentration of pyridoxal phosphate produced insignificant cleavage of cystine in our system so that the formation of cystine trisulphide was due entirely to the enzyme-dependent cleavage of cystine.

TABLE 4. THE SUBSTRATE SPECIFICITY OF CYSTATHIONASE FROM *R. SPHEROIDES*

substrate	concn mM	pyruvate formed nmol
DL-cystathionine	2.0	239
DL-cystine	2.0	251
DL-lanthionine	2.0	200
DL-serine	20.0	263
DL-homocystine	2.0	none
L-homolanthionine	2.0	none
DL-homoserine	20.0	none

Tubes contained substrate, phosphate buffer, pH 7.8, purified cystathionase (0.7 mg of protein) and water in a total volume of 1.0 ml. Incubation was at 37 °C for 1 h and keto-acids were determined as described in the Methods section. No α-ketobutyrate was formed in any of the incubations.

The substrate specificity of cystathionase from R. spheroides

Cystine was the only disulphide tested which was rapidly cleaved by the enzyme. GSSG was significantly degraded (as determined by method 1) but at less than 10 % of the rate for cystine, whilst homocystine was completely inactive as a substrate. These results indicate that the enzyme from *R. spheroides* is a β-cystathionase like those obtained from other micro-organisms (Flavin 1962). The major function of the enzyme in *R. spheroides*, particularly during aerobic growth, is probably not disulphide cleavage but the conversion of cystathionine to homocysteine in the biosynthesis of methionine. Thus, with DL-cystathionine as a substrate the products of the cleavage were homocysteine, pyruvate and ammonia. There was no formation of cysteine or α-ketobutyrate as would be expected if the enzyme were a γ-cystathionase. The

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high activity of this enzyme with DL-lanthionine and DL-serine as substrates and the undetectable activity with L-homolanthionine and DL-homoserine (see table 4) confirm the stated specificity of this enzyme.

Rhodanese-dependent degradation of cystine trisulphide

Rhodanese from *R. spheroides* catalysed the breakdown of cystine trisulphide in the presence of either cyanide or sulphite (see table 5) to give thiocyanate and thiosulphate respectively. With sulphite as acceptor, similar activities were obtained when either the disappearance of sulphite or the formation of thiosulphate was determined (reaction (3)). In order to minimize the non-enzymic degradation of cystine trisulphide which occurs at alkaline pH values, these experiments were done at pH 7.2; this is well below the pH optimum for our purified rhodanese (pH 8.6–9.2). Consequently the enzyme activities shown in tables 5 and 6 are unlikely to represent the maximum rates obtainable with these substrates.

TABLE 5. RHODANESE-DEPENDENT DEGRADATION OF CYSTINE TRISULPHIDE

		product formed (nmol)	
incubation	acceptor substrate	thiosulphate	thiocyanate
1	cyanide	none	610
2	sulphite	280	none

Incubation 1 contained cystine trisulphide (1 μmol), sodium cyanide (2 μmol), tris/HCl buffer, pH 7.2 (100 μmol), rhodanese (35 μg of protein) and water in a total volume of 1.0 ml. Incubation was at 25 °C for 30 min and thiocyanate was determined as described in method 4.

Incubation 2 was identical with incubation 1 except that cyanide was replaced with sodium sulphite (2 μmol). Thiosulphate and sulphite were determined as described in the Methods section. No significant amounts of product were formed in either of the incubations in the absence of enzyme.

TABLE 6. CYSTINE TRISULPHIDE AND THIOSULPHATE AS DONOR SUBSTRATES FOR RHODANESE

			thiocyanate formed
expt	substrate	μmol	nmol
1	cystine trisulphide	0.2	120
2	cystine trisulphide	0.5	320
3	cystine trisulphide	1.0	580
4	sodium thiosulphate	0.5	60
5	sodium thiosulphate	1.0	120
6	sodium thiosulphate	2.0	220

The incubations contained substrate, tris/HCl buffer, pH 7.2 (100 μmol), sodium cyanide (2 μmol), rhodanese (35 μg of protein) and water in a total volume of 1.0 ml. Incubation was at 25 °C for 30 min and thiocyanate was determined as described in method 4.

No significant amounts of thiocyanate were formed in any experiment in the absence of enzyme.

The relative efficiencies of cystine trisulphide and thiosulphate as donor substrates for rhodanese can be seen in table 6. With cyanide as the acceptor the rate of reaction with trisulphide was about five times greater than with thiosulphate at an equivalent concentration. It is therefore clear that rhodanese can degrade trisulphides very effectively given a suitable acceptor substrate. The synthesis of trisulphides by the reverse of reaction (3) could not be demonstrated in incubations of rhodanese even when high concentrations of thiosulphate (40 mM) and cystine (10 mM) were used. It is clear that cystine disulphide is either inactive or a poor acceptor in this system.

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Thiol compounds as acceptor substrates for rhodanese

Rhodanese from *R. spheroides* catalysed the conversion of thiosulphate to sulphite in the presence of L-cysteine or GSH. The activity with the thiol compounds was about 15–20 % of that with cyanide under these conditions (see table 7). The products with cysteine and thiosulphate as substrates are expected to be equal amounts of sulphite and thiocysteine (cf. reaction (4)). We have attempted to demonstrate the formation of thiocysteine by the addition of [^{14}C]iodoacetate to the reaction mixture after incubation. Only small amounts of *S*-carboxymethylthiocysteine, presumably formed by reaction of iodoacetate and thiocysteine, were detected in these mixtures. However, we have not been able to show that the formation of thiocysteine depends on the presence of enzyme. This inability to demonstrate the expected amount of thiocysteine is probably due to the marked instability of the compound. Thiocysteine may decompose to cysteine and free sulphur (Szczepkowski & Wood 1967) or it may react with excess cysteine to form cystine and H_2S (Schneider & Westley 1969). The formation of sulphite in these incubations is however presumptive evidence for the formation of thiocysteine. The possibility of a rhodanese-catalysed synthesis of trisulphide (see Introduction) *in vivo* cannot therefore be excluded.

TABLE 7. ACCEPTOR SUBSTRATES FOR RHODANESE

substrate	μmol	sulphite formed / μmol
L-cysteine	12	0.92
GSH	10	1.48
sodium cyanide	50	6.60

The incubation contained substrate, sodium thiosulphate (32 μmol), tris/HCl buffer, pH 8.7 (100 μmol), rhodanese (25 μg of protein) and water in a total volume of 1.0 ml. Incubation was at 25 °C for 12 min, and the sulphite was determined as described in the Methods section. The values have been corrected for the amount of sulphite (less than 10 % of the total in each expt) formed in the absence of enzyme.

The effect of growth conditions on cystathionase and rhodanese activity in R. spheroides

The specific activities of cystathionase and rhodanese in cells grown under oxygen and in cells grown semi-anaerobically in the light are given in table 8.

The rhodanese activity was only slightly lower in cells grown under oxygen, whilst the cystathionase activity was essentially the same in all samples.

TABLE 8. THE EFFECT OF GROWTH CONDITIONS ON CYSTATHIONASE AND RHODANESE ACTIVITY IN *R. SPHEROIDES*

growth conditions	specific activity / unit/mg: cystathionase	specific activity / unit/mg: rhodanese
semi-anaerobic, light	36, 34, 29, 32	1.5, 1.3, 1.2, 1.7
oxygen, dark	38, 29, 38, 36	1.1, 1.1, 1.2, 1.0

The activities shown are for four separate cultures grown under each set of conditions. Cells were disrupted by sonication and after dilution with two volumes of 0.02 M phosphate buffer, pH 7.4, samples were centrifuged at 400000 *g* for 90 min at 4 °C. Activities were determined in samples which had been dialysed for 16 h at 1 °C against 0.05 M phosphate buffer, pH 7.4. Cystathionase was determined by assay method 2 with L-cystine as substrate. Rhodanese was determined by assay method 4.

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The effect of oxygenation of a culture growing semi-anaerobically in the light on the activity of aminolaevulinate synthetase, cystathionase and rhodanese is shown in figure 5. The fall in aminolaevulinate synthetase activity which is expected under these conditions (Marriott *et al.* 1969) was accompanied by no marked changes in the activity of the other two enzymes. When rhodanese activity was determined in undialysed samples the activity fell by 40 % over the period of oxygenation. This may be due to the accumulation of a dialysable inhibitor of the enzyme. However, this has not yet been investigated. Cystathionase activity could not be measured accurately in undialysed samples due to the accumulation of large amounts of pyruvate in the cells during oxygenation.

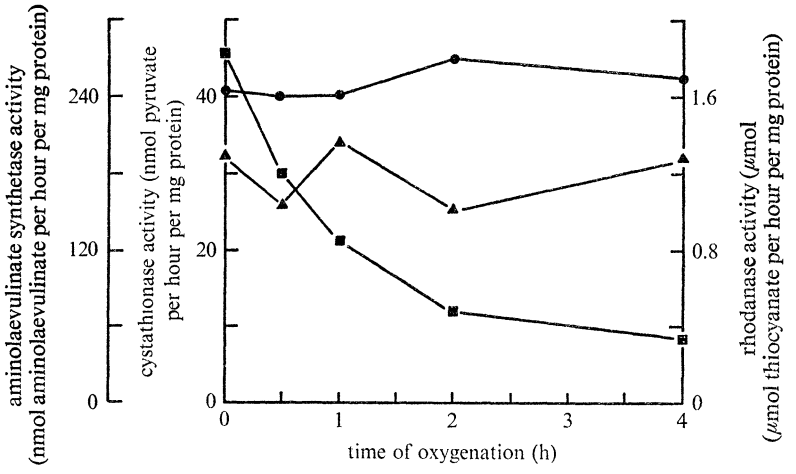

FIGURE 5. The effect of oxygenation of a culture of *R. spheroides* growing semi-anaerobically in the light on the activities of aminolaevulinate synthetase, cystathionase and rhodanese. *R. spheroides* (0.4 mg dry mass/ml of culture) growing semi-anaerobically in the light was oxygenated and samples of cells were harvested at 0.5, 1, 2 and 4 h. Suspensions of washed cells (3 ml, 100 mg dry mass/ml) were disrupted by sonication, 5 ml of 0.02 M phosphate buffer, pH 7.4, were added to each and extracts were centrifuged at 400000 *g* for 90 min. Aliquots of clear supernatants were dialysed against 0.03 M phosphate buffer pH 7.4 for 16 h at 1 °C. Aminolaevulinate synthetase activity (■) was determined in undialysed supernatants. Cystathionase activity (▲, method 2) and rhodanese activity (●, method 4) were determined in dialysed supernatants.

The effect of inhibitors of cystathionase on bacteriochlorophyll synthesis in R. spheroides

If the activity of aminolaevulinate synthetase in *R. spheroides* is controlled by the cellular content of cystine trisulphide (Sandy *et al.* 1975) then inhibition of the biosynthesis of the trisulphide might produce a fall in aminolaevulinate synthetase activity and a subsequent reduction of bacteriochlorophyll synthesis.

Several compounds were tested as possible inhibitors of cystathionase-dependent cleavage of cystine. Compounds which significantly inhibited the formation of pyruvate from cystine and were not substrates are listed in table 9. The most potent inhibitors were β-cyanoalanine and DL-2,3-diaminopropionic acid. The following compounds at mM concentrations were themselves substrates for cystathionase leading to the formation of pyruvate: serine, *S*-methylcysteine, *S*-methylcysteine sulphoxide, *S*-ethylaminocysteine, *S*-carboxymethylcysteine and *S*-sulphocysteine. *S*-Sulphocysteine was the most active substrate tested and was degraded at about three times the rate found for cystine or cystathionine. Glycine, aspartic acid, asparagine and cysteic acid were neither substrates for cystathionase nor inhibitors.

The inhibitors of cystathionase shown in table 9 were also tested as inhibitors of bacteriochlorophyll synthesis in cells growing semi-anaerobically in the light. Compounds (2 mM, except for β-cyanoalanine 0.4 mM) were added to cultures in the exponential phase of growth. Over a period of 6 h bacteriochlorophyll synthesis was inhibited 68 % by 2,3-diaminopropionic acid and less than 12 % by the other compounds. The growth of the cultures during this period was not markedly affected. Moreover, 2,3-diaminopropionic acid (0.1 mM) had no effect on the growth of cells under 100 % oxygen.

TABLE 9. INHIBITORS OF THE CYSTATHIONASE DEPENDENT CLEAVAGE OF CYSTINE

inhibitor	concentration mM	pyruvate formed nmol	activity (%)
none		430	100
β-cyanoalanine	1.0	98	23.0
	2.0	48	12.0
DL-2,3-diaminopropionic acid	1.0	301	70.0
	2.0	206	48.0
DL-2,4-diaminobutyric acid	2.0	275	64.0
DL-methionine	2.0	370	79.0
DL-homoserine	5.0	228	52.0

Incubation mixtures contained purified cystathionase (0.8 mg of protein), L-cystine (2 μmol), phosphate buffer, pH 7.8 (100 μmol), inhibitors as shown and water to a total volume of 1.0 ml. Incubation was at 37 °C for 60 min and the pyruvate was determined as described in the Methods section.

The effect of 2,3-diaminopropionic acid (DAP) (0.1 mM) on bacteriochlorophyll synthesis during adaptation of *R. spheroides* from growth under oxygen to growth under a nitrogen + oxygen (95:5, by vol.) mixture (Gorchein *et al.* 1968) was then examined. We observed that the inhibitor almost completely prevented the synthesis of bacteriochlorophyll, and also inhibited growth by 80 %, over the first 6 h of adaptation. The addition of DAP (0.1 mM) along with methionine (0.1 mM) still prevented bacteriochlorophyll synthesis although growth was now inhibited by only 30 %. The effect of methionine is consistent with DAP acting as an inhibitor of methionine biosynthesis, probably in the conversion of cystathionine to homocysteine by cystathionase. The inhibition of bacteriochlorophyll synthesis by DAP might also be explained by an inhibition of cystathionase resulting in a decreased synthesis of cystine trisulphide.

We also examined the effect of DAP in the presence of methionine on the increase in aminolaevulinate synthetase activity which is observed during adaptation of *R. spheroides* to low oxygen tension (Gorchein *et al.* 1968). The inhibitor did not prevent this increase in aminolaevulinate synthetase if activities were determined in extracts immediately after disruption o the cells. Extracts of untreated cultures showed the expected spontaneous activation of aminolaevulinate synthetase. However, no spontaneous activation occurred in extracts of inhibited cultures indicating the absence of cystine trisulphide. Neither the activity nor the spontaneous activation of aminolaevulinate synthetase was inhibited by DAP (10^{-6} to 10^{-3} M) added to cell-free extracts of normal cells. These results support the idea that DAP has prevented the synthesis of cystine trisulphide.

This effect of DAP on bacteriochlorophyll synthesis during adaptation could not be overcome by the addition of aminolaevulinic acid (0.5 mM). This might be explained by the observation (Lascelles & Hatch 1969) that aminolaevulinic acid added to semi-anaerobic cultures of *R. spheroides* actually inhibits the continued synthesis of bacteriochlorophyll. It is possible that

DAP may produce these changes by effects other than inhibition of cystathionase activity, but this has not been investigated. DAP may cause a deficiency in cellular pyridoxal phosphate since it forms complexes with metal ions and pyridoxal. Whatever the mechanism of its effect it may be potentiated by the known rapid concentration of DAP by living cells (Christensen & Riggs 1956).

Discussion

To our knowledge this paper is the first report of cystathionase activity in the Athiorhodaceae. The enzyme from *R. spheroides* is a β-cystathionase and exhibits similar activities towards thioethers, disulphides, and hydroxyamino acids, in each case producing pyruvate and ammonia. This specificity is consistent with the role of cystathionase in methionine biosynthesis in this organism (Neuberger *et al.* 1973*a*). The ability of cystathionase to convert cystine to its trisulphide indicates a second function for this enzyme in *R. spheroides* and may explain the presence of cystine trisulphide in these cells (Sandy *et al.* 1975). The rhodanese activity of the Athiorhodaceae has been previously studied by Smith & Lascelles (1966) and by Yoch & Lindstrom (1971), although no defined role for this enzyme has been found. The specific activity of the enzyme obtained in the present work agrees well with the findings given in the earlier reports, and the demonstrated ability of rhodanese to degrade cystine trisulphide indicates a function for the enzyme in *R. spheroides*.

A major aim of our current work has been to elucidate the mechanism of the rapid loss of aminolaevulinate synthetase activity observed on oxygenation of a semi-anaerobic culture of *R. spheroides*. We have suggested (Sandy *et al.* 1975) that the loss is due to a displacement of a dynamic equilibrium between a high-activity enzyme (a-form) and a low-activity enzyme (b-form) in the cell. In a semi-anaerobic culture the equilibrium favours the a-form due to the high cellular content of cystine trisulphide. On oxygenation the content of cystine trisulphide is decreased and the conversion of b-form to a-form is thereby prevented. Under these conditions the b-form is favoured. In addition there appears to be a conversion of a-form into b-form.

A postulated scheme for the control of aminolaevulinate synthetase in *R. spheroides* is given in figure 6. It is proposed that the fall in the cellular content of trisulphide on oxygenation is a result of changes in the levels of substrates for cystathionase and possibly rhodanese, rather than changes in the amount of these enzymes (see figure 5). Thus, oxygenation produces a marked disturbance of the sulphur metabolism of *R. spheroides*, leading to a drastic fall in the total glutathione and cystine + cysteine content and the accumulation of homolanthionine (Neuberger *et al.* 1973*a*). This fall in the cellular content of cystine would be expected to reduce the synthesis of trisulphide by cystathionase. The effect of oxygen on the sulphur metabolism was proposed to be the result of its inhibition both of sulphite reduction and of the conversion of homocysteine to methionine. Inhibition of the conversion of sulphite to thiosulphate would lower the cellular content of thiosulphate and might therefore be expected to enhance the degradation of trisulphide by rhodanese. Indeed, the addition of thiosulphate to cultures before oxygenation partially prevents inactivation of aminolaevulinate synthetase whereas sulphite is ineffective (Neuberger *et al.* 1973*a*).

The mechanism of the activation of b-form to a-form by trisulphide is unknown. The trisulphide may modify the enzyme by transfer of sulphur or it may act catalytically in the formation of an intramolecular disulphide bridge. Activation of b-form enzyme by disulphides has been achieved by incubation with an activating enzyme (Tuboi & Hayasaka 1972), although

the mechanism is not fully understood. It is of interest, however, that the chromatographic behaviour of this enzyme and cystathionase on DEAE Sephadex (figure 2) are similar, although the apparent molecular mass of the activating enzyme (90000) is greater than that of cystathionase (55000). If a-form enzyme contains a sulphane sulphur atom then the conversion of a-form to b-form during oxygenation may involve the transfer of the sulphane sulphur to a thiophile (e.g. sulphite) by the action of rhodanese. Further, conversion of a-form to b-form by this mechanism and removal of sulphane sulphur from cystine trisulphide, may both occur continually in the cell, in which case oxygenation need only influence the rate of synthesis of cystine trisulphide and not its rate of degradation.

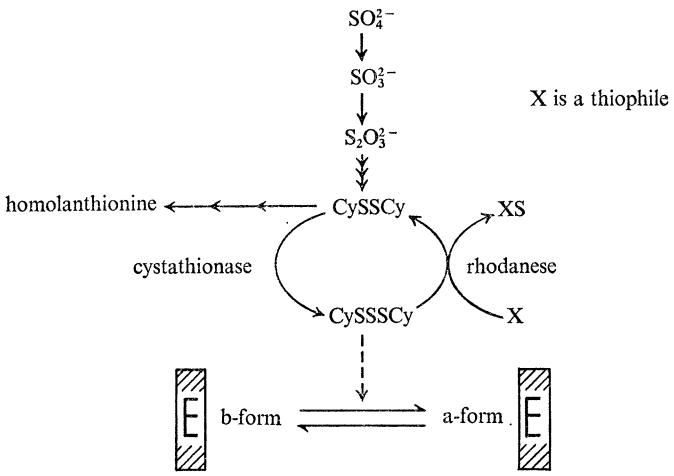

FIGURE 6. Postulated scheme for the control of aminolaevulinate synthetase in *R. spheroides*.

While the proposed scheme accommodates the available data it is clear that many of its aspects are speculative. These aspects are, however, open to further experimental investigation.

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Note added in proof (23 *September* 1975)

In the experiments described above, and in earlier work on this subject (Neuberger *et al.* 1973*a*), the system studied was one in which bacteriochlorophyll synthesis in semi-anaerobic cultures of *R. spheroides* was terminated by bubbling with pure oxygen. Recently we have extended this work to an investigation of the effect of bubbling with nitrogen-oxygen mixtures containing 10% and 20% oxygen (by vol.). Under these conditions also, bacteriochlorophyll synthesis ceases abruptly, and during the first hour there is approximately a 50% loss of 5-aminolaevulinate synthetase activity. There are, however, no marked changes in the intracellular concentrations of GSH+GSSG, cysteine+cystine or cystine trisulphide. It would therefore appear that the loss of enzyme activity observed under relatively low oxygen tensions is not mediated through gross changes in the cellular content of sulphur compounds, but by some as yet unknown mechanism probably involving the conversion of a-form enzyme into

b-form enzyme. Thus we have found that the low-activity enzyme extracted from cells harvested after 1 h of aeration, is activated by cystine trisulphide to the same extent as the b-form enzyme from oxygenated cells. The sulphur content and the thiol-disulphide status of the a-form and the b-form of the enzyme are now under investigation.

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Discussion

M. R. Moore, (*Department of Materia Medica, University of Glasgow, Stobhill General Hospital, Glasgow G*21 3*UW*). Do you have evidence for sulphur incorporation into the trisulphide or persulphide to validate one of your hypotheses?

J. D. Sandy. No. The sulphur content of the different forms of purified aminolaevulinate synthetase has not yet been determined.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES