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Control of 5-Aminolaevulinate Synthetase Activity in *Rhodopseudomonas spheroides*

THE INVOLVEMENT OF SULPHUR METABOLISM

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1. The 'initial' 5-aminolaevulinate synthetase activity, that is the activity observed immediately after cell disruption, in extracts prepared from unharvested semi-anaerobically grown *Rhodopseudomonas spheroides*, was twice that observed under the same assay conditions in extracts prepared from harvested cells. 2. The effect of oxygenation of a culture on the 'maximum' aminolaevulinate synthetase activity, that is the activity observed 1h after disruption of harvested cells, is markedly influenced by the contents of the growth medium. Oxygenation of organisms for 1h in the medium in which they have grown produces an 80–90% decrease in maximum activity, whereas similar treatment of organisms resuspended in fresh medium produces less than a 40% decrease. 3. This protective effect of fresh medium is absolutely dependent on the presence of sulphate. When cells are suspended in sulphate-deficient fresh medium, the maximum activity falls by 65–75% even without oxygenation. A high maximum activity is regenerated when sulphate is resupplied. 4. When organisms are oxygenated in the medium in which they have grown, the cellular contents of GSH+GSSG and cysteine+cystine fall very markedly and homolanthionine is formed. Both the fall in aminolaevulinate synthetase activity and the changes in sulphur metabolism are largely prevented by the addition of compounds which stimulate synthesis of cysteine *de novo* or inhibit the conversion of cysteine S into homocysteine S. 5. The maximum aminolaevulinate synthetase activity was directly proportional to the GSH+GSSG content of all cell preparations. In glutathione-depleted extracts the 'low'-activity enzyme could be re-activated *in vitro* by the addition of GSH, GSSG, cysteine or cystine, whereas in extracts with a high glutathione content the 'high'-activity enzyme was unaffected by these sulphur compounds. 6. The activation of low-activity enzyme with exogenous sulphur compounds was prevented by excluding air or by adding NADH. Studies with purified enzyme indicate that sulphur compounds do not interact directly with the enzyme, but that their effect is mediated by a number of other endogenous factors.

When air is bubbled through a culture of *Rhodopseudomonas spheroides* growing semi-anaerobically in the light, bacteriochlorophyll synthesis ceases immediately (Cohen-Bazire *et al.*, 1957) and there is no accumulation of aminolaevulinate or other intermediates of the bacteriochlorophyll pathway (cf. Lascelles, 1968). It is now clear that oxygen inhibits this pathway at at least two points. Lascelles & Altshuler (1969) obtained evidence that oxygen inhibits the first step of the magnesium branch of the pathway, and this has been confirmed by the assay of magnesium–protoporphyrin chelatase activity in whole cells (Gorchein, 1972). The first step of the pathway, catalysed by aminolaevulinate synthetase, is also inhibited by oxygen (Higuchi *et al.*, 1965; Gorchein *et al.*, 1968), although the mechanism of this effect is not clear.

Lascelles (1960) showed that high oxygen partial pressures suppress the synthesis of aminolaevulinate synthetase; however, the immediate and rapid fall in aminolaevulinate synthesis observed on aeration of a culture requires that some process of direct inhibition or rapid degradation of the enzyme must also be involved. Lascelles (1968) has suggested that oxygenation results in the direct inhibition of aminolaevulinate synthetase activity by raised concentrations of free haem produced as a result of inhibition of the magnesium branch of the pathway. This hypothesis is based on the finding (Gibson *et al.*, 1961; Burnham & Lascelles, 1963) that the enzyme is inhibited *in vitro* by μM concentrations of haemin; it has, however, not been possible to demonstrate the postulated increase in the intracellular content of free haem on oxygenation.

Marriott (1968) found that oxygenation of a culture of *R. spheroides* growing semi-anaerobically in the light resulted in a rapid 8–10-fold decrease in aminolaevulinate synthetase activity. This marked decrease could only be demonstrated, however, if extracts of freshly sonicated cells were left for 1h at 4°C before assay of the enzyme. If the enzyme was

assayed immediately after cell disruption, culture oxygenation resulted in less than a 2-fold decrease in activity. This was explained by the observation that the enzyme undergoes spontaneous activation in fresh extracts of semi-anaerobically grown cells and that this activation process is abolished by oxygenation of the culture before harvesting. It was therefore suggested that the spontaneous activation of the enzyme *in vitro* might be a direct reflection of the control mechanism *in vivo* for the enzyme. This idea was supported by subsequent work (Marriott *et al.*, 1969, 1970) in which the activation process was shown to be dependent on electron transport and to be controlled by a number of endogenous low-molecular-weight compounds. It was concluded that for activation of the enzyme to occur *in vitro* some endogenous factor has to be oxidized via the electron-transport chain.

Tuboi & Hayasaka (1972*a*) have shown that a partially purified extract of aminolaevulinate synthetase from *R. spheroides* can be activated *in vitro* by the addition of any of a number of disulphide compounds. It has been found that activation with sulphur compounds is dependent on an activating enzyme, which has been partially purified from semi-anaerobically grown *R. spheroides* (Tuboi & Hayasaka, 1972*b*).

In the present paper we describe results that show that the suppression of the spontaneous activation of aminolaevulinate synthetase in cell-free extracts by oxygenation of the culture can be directly attributed to the effect of oxygen on the metabolism of sulphur compounds of low molecular weight. Thus during 1h of oxygenation the intracellular concentrations of GSH+GSSG and 'cysteine+cystine' fall to less than 10% of the values before oxygenation. The low-activity enzyme in glutathione-depleted extracts does not increase in activity spontaneously, but it can be activated by the addition of GSH, GSSG, cysteine or cystine to the extract. The mechanism by which culture oxygenation disturbs the sulphur metabolism of *R. spheroides* and the mechanism of the activation of low-activity aminolaevulinate synthetase with sulphur compounds have now been investigated.

Materials and Methods

Materials

Chemicals. CoA, ATP, NADH and GSH were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K.; DL-homoserine, GSSG and DEAE-cellulose were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.; *meso*-cystine was from Fluka AG, Buchs, Switzerland; DL-homocysteine thiolactone was from Mann Research Laboratories, New York, N.Y., U.S.A.; L-cystathionine was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; [^{35}S]sulphate was from The Radiochemical Centre, Amersham, Bucks., U.K. L-Homolanthionine was kindly supplied by Dr. H. T. Huang, International Minerals and Chemical Corp., Libertyville, Ill., U.S.A. Phosphate buffers were prepared from KH_2PO_4 and K_2HPO_4. Other chemicals were commercial preparations of the purest grade available.

Preparation of 'acid-treated' cystine. L-Cystine was dissolved in 6M-HCl and kept at 110°C for 16h in a sealed evacuated tube. The acid was removed under vacuum and the residue dissolved in water to make a 10mM solution.

Growth and harvesting of organisms

Rhodopseudomonas spheroides (N.C.I.B. 8253) was grown semi-anaerobically in the light in 3-litre conical flasks at 32–34°C for 16–24h in the malate–glutamate medium (medium S) described by Lascelles (1956). The flasks were filled almost to the top and were stoppered with cotton-wool. No special precautions were taken to make the conditions strictly anaerobic. Illumination was supplied and temperature maintained by 60W bulbs placed 30–40cm from the flasks. Unless otherwise stated, organisms were harvested by centrifuging for 10min at 15000*g* at 4°C in the late exponential phase of growth (0.4–0.6mg dry wt./ml), washed in 0.05M-phosphate buffer, pH7.0, then resuspended to about 100mg dry wt./ml in the same buffer and finally were stored at −20°C until required.

Growth of organisms on [^{35}S]sulphate. R. spheroides was grown as described above, except that normal medium S was replaced with medium S containing 25% of the normal complement of $MgSO_4$. At the time of inoculation about 6mCi of [^{35}S]sulphate (carrier-free) was added to the medium.

Procedure for oxygenation experiments. In experiments involving oxygenation of *R. spheroides* in culture, cells were grown and harvested as described above except that harvesting was performed at 22–24°C instead of at 4°C. Freshly harvested organisms were used in all experiments. The test medium, contained in a 3-litre conical flask filled almost to the top, was brought to 32–34°C and the cells were suspended in it to a concentration of 0.3–0.45mg dry wt./ml. The culture was incubated under normal growth conditions for 1h before the start of oxygenation.

Preparation of cell-free extracts. Cell suspensions at about 100mg dry wt./ml were disrupted by irradiation in an MSE 50W Ultrasonic Disintegrator operated at maximum output for 2min at 0°C. Immediately afterwards the extract was centrifuged at 4°C for 10min at 25000*g* and the supernatant was used immediately or stored at −20°C until required.

Determinations

The dry weight of organisms was calculated from a calibration curve by measurement of the E_{680} of cell suspensions (Cohen-Bazire *et al.*, 1957). Protein was determined by the method of Lowry *et al.* (1951) with bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) as standard.

Assay of aminolaevulinate synthetase activity. The method was the same as that described by Lascelles (1960) except that GSH was omitted from the substrate mixture. Extracts containing 0.05–1.0mg of protein were added to the substrate mixture in a total volume of 1.0ml and incubated at 37°C for 30min. Aminolaevulinate formed was determined by the method of Mauzerall & Granick (1956). Activity is expressed as nmol of aminolaevulinate formed/h per mg of protein.

Assay of aminolaevulinate synthetase activity in extracts of unharvested cells. A portion (1.25ml) of a culture of *R. spheroides* growing semi-anaerobically in the light (about 0.65mg dry wt. of cells) was sonicated for 1min as described above. Some (0.5ml) of the sonicated material was centrifuged at 25000*g* for 20min and the supernatant assayed for protein (with an appropriate culture-filtrate blank). The remaining 0.75ml of sonicated material was added to 0.25ml of aminolaevulinate synthetase substrate mixture. Incubation was at 37°C for 2h and the aminolaevulinate formed was measured as described above with an appropriate culture-filtrate blank.

Assay of succinate thiokinase activity. Succinate thiokinase (EC 6.2.1.5) was assayed exactly as described by Marriott *et al.* (1969).

Other methods

Extraction of amino acids from R. spheroides. Suspensions of *R. spheroides* (1.0ml, about 100mg dry wt.) were extracted with three successive 4.0ml portions of trichloroacetic acid (10%, w/v). After centrifugation (4°C, 10min, 25000*g*) the clear supernatants were combined and extracted with four successive 20ml portions of ether. The aqueous phase was neutralized with NaOH and applied to a column (1cm×7cm) of Dowex 50 (X2; H^+ form; 200–400 mesh) that had been previously equilibrated in water. The column was then washed with water (40ml) and eluted with 2M-NH_3. The first 6ml of the NH_3 eluate was collected and freeze-dried.

Performic acid treatment of amino acid extracts. Freeze-dried extracts were dissolved in a minimum (100–250μl) of performic acid [formic acid (98–100%) $-H_2O_2$ (100vol.), 9:1, v/v] and left at room temperature for 1h. After addition of 2ml of water the mixture was freeze-dried. This procedure gave a quantitative conversion of GSH+GSSG into the cysteic acid derivative and cysteine+cystine into cysteic acid.

High-voltage electrophoresis. The technique was essentially that described by Atfield & Morris (1961). Electrophoresis was performed on Whatman 3MM paper (22cm×56cm) in 2.5% (v/v) formic acid–7.8% (v/v) acetic acid, pH2.0, at 3kV and 65mA for 30min. The Whatman 3MM paper was washed with 1M-HCl and water before use.

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

Determination of GSH+GSSG and cysteine+cystine. The amino acid fraction from 100mg dry wt. of *R. spheroides* was treated with performic acid and the freeze-dried extract was dissolved in 250μl of water. Three samples (10, 25 and 50μl) were fractionated by high-voltage electrophoresis at pH2.0. On the same electrophoresis strip was applied a range (10–100nmol) of standard solutions of (i) the performic acid-oxidized product of GSH and (ii) cysteic acid. After electrophoresis the paper was dried and stained with ninhydrin reagent [0.4% (w/v) in acetone containing 0.1% (v/v) pyridine]. By comparing visually the intensity of ninhydrin colour for unknowns and standards, a semi-quantitative estimate of GSH+GSSG and cysteine+cystine (as cysteic acid) was obtained.

Preparation and use of Raney nickel catalyst W-2. Raney nickel catalyst W-2 was used for desulphurization of thioethers. The catalyst was prepared from nickel–aluminium alloy (BDH Chemicals Ltd., Poole, Dorset, U.K.) as described by Mozingo (1955). Freeze-dried extracts containing thioethers were dissolved in 1.0ml of water and a small spatula end of catalyst was added. The tube was sealed and kept at 120°C for 3h. The supernatant was removed and the catalyst washed with water (3×0.5ml) and the washings were added to the supernatant. This combined solution was centrifuged (1000*g*, 5min), the supernatant was extracted with three successive 0.5ml portions of chloroform containing 8-hydroxyquinoline (0.1%, w/v) and the aqueous phase was freeze-dried.

Ultrafiltration of protein extracts. This was performed with a C50 ultrafiltration system from ChemLab, Ilford, Essex, U.K. Membranes were type UM-10 from Amicon Corp., Lexington, Mass., U.S.A.

Purification of 'low'-activity aminolaevulinate synthetase from oxygenated R. spheroides

All steps were done at 0–4°C.

Step 1. A culture of *R. spheroides* growing semi-anaerobically in the light was oxygenated for 1h before harvesting. Organisms (about 500mg dry wt.) were disrupted by sonication at about 100mg dry

wt./ml, centrifuged immediately at 25000*g* and 4°C for 15min and the cell-free supernatant was removed. This preparation is hereafter termed crude low-activity enzyme.

Step 2. The supernatant was diluted in 0.05M-phosphate buffer, pH7.0, to about 2mg of protein/ml and was centrifuged at 105000*g* for 2h. The preparation was separated into a straw-coloured upper supernatant, a pigmented lower supernatant and a solid pellet.

Step 3. The upper supernatant from step 2 was loaded on a column (1.6cm×10cm) of DEAE-cellulose equilibrated with 0.001M-phosphate buffer, pH7.0. The column was eluted at about 0.5ml/min with 0.001M-phosphate buffer, pH7.0 (25ml), and then with 0.5M-phosphate buffer, pH7.0. The aminolaevulinate synthetase was eluted with the 0.5M-phosphate buffer front and over 85% of the applied activity was recovered in the first 15ml collected.

Step 4. The combined active fractions from step 3 were concentrated to about 3ml by ultrafiltration and the extract was applied to a column (2.25cm×60cm) of Sephadex G-100 previously equilibrated in 0.025M-phosphate buffer, pH7.0. Elution was carried out at about 12ml/h and 3ml fractions were collected.

Preparation of the 'pellet factor' from R. spheroides

The pellet fraction from step 2 of the purification procedure described above was suspended in 2ml of 0.05M-phosphate buffer, pH7.0, and stored at −20°C.

Preparation of crude 'high'-activity enzyme

A cell-free extract (at about 40mg of protein/ml) was prepared from *R. spheroides* that had been grown under semi-anaerobic conditions as described above. The extract was diluted in 0.05M-phosphate buffer, pH7.0, to about 10mg of protein/ml and stirred gently at 4°C for 90min.

Results

Effect of harvesting on aminolaevulinate synthetase activity

In the assay of bacterial enzymes organisms are normally harvested by centrifugation, the cells are disrupted and appropriate fractions of the extracts are taken for assay. If an enzyme activity is stable in the crude extract it is generally assumed that the process of harvesting the cells and preparing the extract does not markedly alter the activity of the enzyme, and also that any alterations in activity that occur *in vivo* are reflected in similar alterations in the activity measured *in vitro*. The activity of aminolaevulinate synthetase in crude extracts of semi-anaerobically grown *R. spheroides* spontaneously increases 5–6-fold on storage, but only in the presence of air or light (Marriott *et al.*, 1970). A similar activation was observed by Warnick & Burnham (1971) on addition of potassium ferricyanide to extracts and it would therefore appear that the activity of the enzyme in crude extracts is increased under conditions leading to the oxygenation of some endogenous compound. It therefore seemed possible that the activity of the enzyme might also be affected by the harvesting process, which is carried out under dark anaerobic conditions.

To investigate this, aminolaevulinate synthetase was assayed in extracts of semi-anaerobically grown *R. spheroides* before and after the cells were harvested. Assays were performed in triplicate on two different cultures (Table 1). In every case the effect of harvesting was to lower the activity of the enzyme by 40–60%.

This finding provides an explanation for the low 'initial' activity observed immediately after disruption of harvested cells (Marriott, 1968). It would appear that the high-activity form of the enzyme, present in the cell during semi-anaerobic growth, is converted into a low-activity form as a result of the reducing conditions attained in the cell during harvesting. That harvesting results

Table 1. *Effect of harvesting on aminolaevulinate synthetase activity*

The method for assay of aminolaevulinate synthetase before harvesting is described in the Materials and Methods section. To determine the effect of harvesting, three 1.5ml samples of each culture grown semi-anaerobically in the light were centrifuged at 25000*g* and 4°C for 5min. The culture supernatants were retained and the cells washed in 0.05M-phosphate buffer, pH7.0, and again centrifuged as above. The cells were then resuspended in the culture supernatant and the enzyme activity was assayed as described for unharvested cells.

Culture no.	Aminolaevulinate synthetase activity (units/mg of protein)	
	Before harvesting	After harvesting
1	143	74
	184	82
	200	87
2	124	65
	117	62
	97	58

in more reducing conditions in the cell is supported by the work of Cohen-Bazire *et al.* (1957) on the effects of oxygen and light intensity on the rate of pigment synthesis in these organisms. Further, Wimpenny & Firth (1972) have reported a marked fall in the ratio of NAD^+ to NADH when *Klebsiella aerogenes* is harvested by centrifugation.

The mechanism by which the enzyme is inactivated during harvesting is presumed to be different from that which operates during oxygenation of a culture. *R. spheroides* contains a low-molecular-weight activator of aminolaevulinate synthetase, which is inactivated by both reducing and strongly oxidizing conditions (Neuberger *et al.*, 1973). This activator may be inactivated by reduction during harvesting and by oxidation during oxygenation of a culture. A more complete discussion of this point is given in the following paper (Neuberger *et al.*, 1973). The spontaneous activation of the enzyme *in vitro* probably represents a re-activation of the low-activity form back to the high-activity form present in the cell before harvesting. The most accurate measure of the activity of the enzyme in the growing culture would seem to be that obtained after allowing the enzyme to become activated spontaneously *in vitro*. For this reason all values given below for aminolaevulinate synthetase activity in crude extracts refer to the activity obtained after storage of the extract for 1h at 4°C under conditions for spontaneous activation. This activity will be referred to as 'maximum' aminolaevulinate synthetase activity.

Effect of culture oxygenation on maximum aminolaevulinate synthetase activity

Marriott *et al.* (1969) observed an 80–90% fall in maximum aminolaevulinate synthetase activity on bubbling O_2 for 1h through a culture of *R. spheroides* growing semi-anaerobically in the light. Oxygenation was performed in the late exponential phase of growth.

To investigate the mechanism of this inactivation process we studied the effect of oxygenation on the maximum aminolaevulinate synthetase activity of cultures at various stages of growth. A 80–90% fall occurred at all stages of growth above that corresponding to 0.15mg dry wt. of cells/ml of culture. In the early growth phase, however, less than a 50% fall in activity was observed during 1h of oxygenation. When harvested cells were resuspended (see the Materials and Methods section) at about 0.45mg dry wt./ml in fresh medium S, less than a 50% fall in maximum activity occurred during 1h of oxygenation. A comparison of the inactivation curves for cells suspended in spent medium S (i.e. the medium in which they had grown) and in fresh medium S is shown in Fig. 1.

To determine which constituents of the fresh medium were required for this protection, the effect of oxygenation (1h) on the maximum activity of cells suspended in a variety of different media was studied (Table 2). There was less than a 30% loss on oxygenation in phosphate buffer alone. If malate, glutamate or both of these constituents were added there was a 65–85% loss; however, if $MgSO_4$ was also included, then the loss was less than 35%. $MgCl_2$ could not replace $MgSO_4$ in this protective effect.

When organisms were suspended in fresh medium S containing no $MgSO_4$ a rapid 70–80% fall in

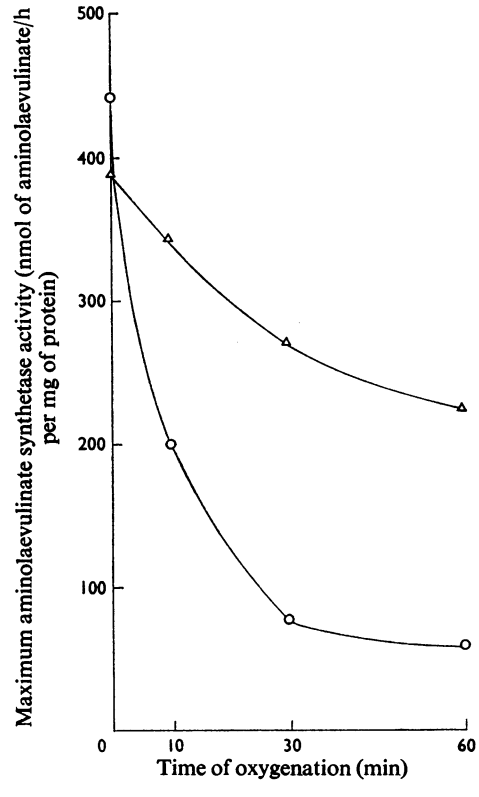


Fig. 1. *Effect of oxygenation on the maximum aminolaevulinate synthetase activity of cells suspended in spent medium S and fresh medium S*

R. spheroides growing semi-anaerobically in the light was harvested, washed and resuspended at about 0.45mg dry wt./ml in spent medium S or fresh medium S and incubated under normal growth conditions. After 1h, oxygenation was started and samples were taken at intervals for assay of maximum aminolaevulinate synthetase activity. ○, Cells suspended in spent medium S; △, cells suspended in fresh medium S.

Table 2. *Effect of oxygenation on the maximum aminolaevulinate synthetase activity of cells suspended in a variety of media*

R. spheroides growing semi-anaerobically in the light was harvested, washed and resuspended at about 0.45mg dry wt./ml in potassium phosphate buffer, pH6.8, containing the additional constituents described in the table. All medium constituents were added at the concentrations present in medium S of Lascelles (1956) and media were adjusted to pH6.8 with NaOH where necessary. $MgCl_2$ was added to a final concentration of 0.8mM. After 1h under normal growth conditions a sample was taken, the culture was oxygenated for 1h and another sample taken. Both samples were harvested, and the cells were washed and assayed for maximum aminolaevulinate synthetase activity.

Additional constituents of medium	Maximum aminolaevulinate synthetase activity (units/mg of protein)	
	Before oxygen	After oxygen
None	255	185
DL-Malate	250	45
Sodium-L-glutamate	224	73
DL-Malate, sodium-L-glutamate	255	72
DL-Malate, $MgSO_4$	305	205
Sodium-L-glutamate, $MgSO_4$	268	175
Sodium-L-glutamate, DL-malate, $MgSO_4$	275	198
Sodium L-glutamate, DL-malate, $MgCl_2$	236	52

maximum activity occurred even without oxygenation (Fig. 2). This process was reversible, since if $MgSO_4$ was supplied to the culture after 2h, then the maximum activity was partially regenerated over the following 2h. $MgCl_2$ produced no effect if added instead of $MgSO_4$. It is clear that under these conditions the availability of sulphate in the medium directly determines the maximum enzyme activity.

Effect of supplements in the medium on the inactivation of maximum aminolaevulinate synthetase by culture oxygenation

To clarify the relationship between sulphur metabolism and aminolaevulinate synthetase activity in *R. spheroides* we studied the effect of compounds related to the sulphur pathway on the inactivation of the maximum activity on culture oxygenation

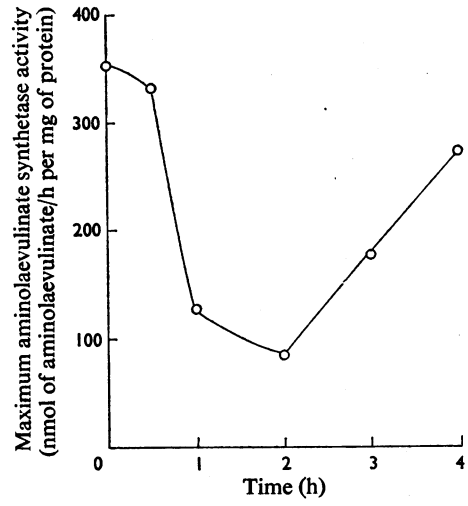


Fig. 2. *Effect of sulphate-deficient medium S on the maximum aminolaevulinate synthetase activity*

R. spheroides growing semi-anaerobically in the light was harvested, washed and resuspended at about 0.45mg dry wt./ml in fresh medium S containing no $MgSO_4$. The culture was incubated under normal growth conditions and after 2h $MgSO_4$ (0.8mM) was added to the culture. Samples were taken at intervals throughout the experiment and the cells were harvested, washed and assayed for maximum aminolaevulinate synthetase activity.

(Table 3). A number of compounds, when added to the culture 1h before the start of oxygenation, significantly protected the maximum activity. The effective compounds were cysteine, cystine, methionine, threonine, homocysteine thiolactone and sodium thiosulphate. Under identical conditions, sulphate, sulphite, sulphide and homoserine were ineffective. Homoserine reversed the protective effect of threonine but not that of methionine. The high activities observed before and after oxygenation with added cysteine or cystine can be explained in part by the carry-over of these compounds into the assay tube and subsequent activation of the enzyme *in vitro* (see below).

Effect of culture oxygenation on the metabolism of sulphur compounds of low molecular weight in R. spheroides

R. spheroides was grown in medium S containing [^{35}S]sulphate (see the Materials and Methods section). Labelled cells were harvested and resuspended at about 0.45mg dry wt./ml in 3 litres of non-radioactive spent medium S; a 1-litre sample (culture 1) was harvested immediately and the remainder was

Table 3. *Effect of supplements to the medium on the inactivation of maximum aminolaevulinate synthetase by culture oxygenation*

R. spheroides was grown semi-anaerobically in the light to about 0.45mg dry wt./ml. Supplements were added to the culture at the final concentrations indicated and the culture was kept under normal growth conditions for 1h. A sample was then taken, the culture oxygenated for 1h and another sample taken. Both samples were harvested, and the cells were washed and assayed for maximum aminolaevulinate synthetase activity.

Supplement	Concentration (mM)	Maximum aminolaevulinate synthetase activity (units/mg of protein) Before oxygen	After oxygen
None		385	52
$MgSO_4,7H_2O$	0.8	395	55
DL-Cysteine–HCl	5	545	375
DL-Cystine	5	410	235
DL-Methionine	1	302	167
DL-Threonine	1	220	149
DL-Homoserine	2	327	42
DL-Methionine + DL-homoserine	1 + 2	242	146
DL-Threonine + DL-homoserine	1 + 2	255	35
DL-Homocysteine-thiolactone	1	282	192
$Na_2S,9H_2O$	1	237	42
$Na_2SO_3,7H_2O$	1	246	34
$Na_2S_2O_3,5H_2O$	1	240	136

divided equally between two flasks under normal growth conditions. One flask (culture 2) was left untreated and the other (culture 3) was oxygenated for 1h. Both cultures were then harvested and a sample of the three cell preparations was extracted for amino acids (see the Materials and Methods section). Extracts were fractionated on high-voltage electrophoresis, pH2.0, and a radiograph of the electrophoretogram was made by contact with Kodirex X-ray film (Kodak, London, W.C.2, U.K.) in a lead-backed exposure holder for 1–3 days.

Oxygenation produced a striking change in the concentrations of the major amino-sulphur compounds of the cell. In cultures 1 and 2, which had been treated under semi-anaerobic growth conditions, over 90% of the total sulphur in the amino acid fraction was contained in a single compound that has been identified as GSSG. In culture 3, however, which had been oxygenated for 1h, only traces of GSSG were detected; instead over 80% of the total sulphur was contained in a single compound that has been identified as homolanthionine. The isolation of glutathione almost entirely in the oxidized form can be explained by oxidation of GSH during extraction. It seems likely, however, that, in common with other tissues studied (Knox, 1960), GSH predominates over GSSG 10–100-fold in *R. spheroides* grown under semi-anaerobic conditions.

Identification of GSSG and homolanthionine in extracts of R. spheroides

Both of the major radioactive compounds described above were prepared essentially pure by elution from the pH2.0 electrophoretogram. Minor ninhydrin-positive contaminants were resolved from the labelled compounds by repeated electrophoresis of extracts at pH2.0.

The radioactive compound isolated from semi-anaerobic cells was identified as GSSG on the basis of the following results. (i) It behaved identically with authentic GSSG on electrophoresis at pH2.0. Mobility relative to alanine was +0.46. (ii) It was converted by performic acid treatment into a compound that behaved identically with the performic acid-oxidized product of GSSG on electrophoresis at pH2.0, with mobility relative to alanine of −0.23. (iii) Acid hydrolysis (6M-HCl, 110°C, 16h) of the isolated performic acid-oxidized product resulted in its conversion into three new ninhydrin-positive compounds. These compounds were indistinguishable from glutamic acid, glycine and cysteic acid on electrophoresis at pH2.0 and on t.l.c. on cellulose in butan-1-ol–acetic acid–water (12:3:5, by vol.). Electrophoretic mobilities relative to alanine were glycine +1.10, glutamic acid +0.67 and cysteic acid −0.52. Chromatographic R_F values were glycine 0.25, glutamic acid 0.28 and cysteic acid 0.05.

The radioactive compound isolated from oxygenated cells was identified as homolanthionine on the basis of the following results. (i) It behaved identically with authentic homolanthionine on high-voltage electrophoresis at pH2.0. Mobility relative to alanine was +0.82. (ii) It was indistinguishable from authentic homolanthionine on t.l.c. on cellulose in the following three solvents: (*a*) butanol–acetic acid–water (12:3:5, by vol.) ($R_F = 0.16$); (*b*) aq. 80% (v/v) phenol–conc. NH_3 (sp.gr. 0.88) (200:1, v/v) ($R_F = 0.33$); (*c*) methanol–water–pyridine (20:5:1, by vol.) ($R_F = 0.20$). (iii) On performic acid oxidation it was converted into a compound which behaved identically with authentic homolanthionine sulphone on high-voltage electrophoresis at pH2.0,

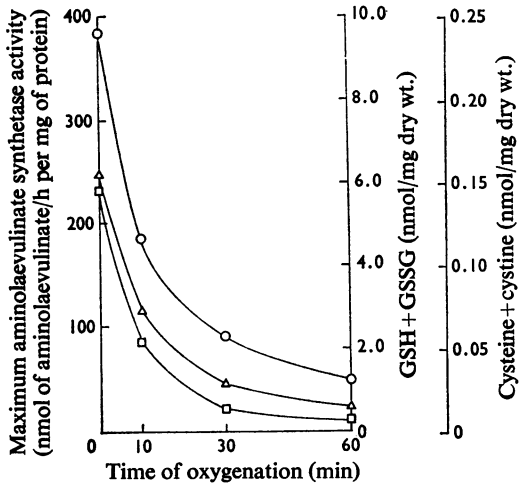


Fig. 3. *Effect of oxygenation of a culture on the maximum aminolaevulinate synthetase activity and the contents of GSH+GSSG and cysteine+cystine*

R. spheroides growing semi-anaerobically in the light at about 0.45mg dry wt./ml was oxygenated and samples were taken at intervals for assay of maximum aminolaevulinate synthetase activity (○), GSH+GSSG (△) and cysteine+cystine (□).

Mobility relative to alanine was +0.65. (iv) On treatment with Raney nickel catalyst W-2, it was converted into a single new ninhydrin-positive compound. The new compound was indistinguishable from authentic α-aminobutyric acid on high-voltage electrophoresis at pH 2.0. Mobility relative to alanine was +0.89. The new compound co-chromatographed with authentic α-aminobutyric acid on t.l.c. on cellulose in the three solvents detailed in (ii). R_F values in the three solvents were (*a*) 0.44, (*b*) 0.69 and (*c*) 0.59 respectively.

Relationship of the GSH+GSSG and cysteine+cystine content of extracts to the maximum aminolaevulinate synthetase activity

The experiments described above indicated a direct relationship between the GSH+GSSG concentration and the maximum activity of extracts. To study this further a method for the semi-quantitative determination of GSH+GSSG and cysteine+cystine in extracts was developed (see the Materials and Methods section).

When a culture of *R. spheroides* growing semi-anaerobically in the light was oxygenated and samples were taken at intervals for determination of amino-sulphur compounds and for assay of the maximum enzyme activity (Fig. 3), the fall in enzyme activity was accompanied by exactly parallel changes in the contents of GSH+GSSG and cysteine+cystine.

When cell preparations from each of the oxygenation experiments detailed in Tables 2 and 3 were similarly assayed, in every case the content of amino-sulphur compounds was directly related to the maximum aminolaevulinate synthetase activity. Thus in samples taken before oxygenation, in which the maximum activity was high (220–350 units/mg of protein), the content of amino-sulphur compounds was high and similar to the values shown in Fig. 3 at zero time. In samples taken after oxygenation, in which the maximum activity was low (34–75 units/mg of protein), the contents of amino-sulphur compounds were low and approximated to the values shown in Fig. 3 at 60min. In all experiments (Tables 2 and 3) where the enzyme activity was maintained at a relatively high value during oxygenation (136–205 units/mg of protein) the contents of the sulphur compounds also remained at the high values observed before oxygenation.

The GSH+GSSG and cysteine+cystine contents were measured in cell preparations taken from the experiment described in Fig. 2. The fall in maximum activity during resuspension in sulphate-deficient medium S was accompanied by a parallel decrease in the concentration of the sulphur compounds. Further, the regeneration of activity on resupplying sulphate was associated with a similar regeneration of the intracellular contents of the sulphur compounds.

The contents of these sulphur compounds were measured in an extract of semi-anaerobic cells at intervals during the spontaneous activation of aminolaevulinate synthetase. The high initial contents did not alter significantly during a 90-min activation period, whereas the enzyme activity increased from 73 to 325 units/mg of protein. Therefore, although spontaneous activation of the enzyme could only be demonstrated in extracts with a high content of amino-sulphur compounds, it was clear that the compounds were not consumed in the activation process.

Effect of sulphur compounds on aminolaevulinate synthetase activity in vitro

The results presented above indicated a causal relationship between the contents of amino-sulphur compounds and the maximum activity of extracts. If the low maximum activity in glutathione-depleted extracts was a direct consequence of this depletion, then addition of glutathione might be expected to re-activate the enzyme.

To investigate this, crude low-activity enzyme was prepared from semi-anaerobically grown cells that had been oxygenated for 1h in spent medium S before harvesting. The specific activity

of this preparation was always in the range 30–50 aminolaevulinate synthetase units/mg of protein. The effect of GSH, GSSG, cysteine and cystine on the activity of this preparation was investigated and the effect of GSSG is shown in Fig. 4. At low concentrations of GSSG the activations observed were time-dependent and resembled the spontaneous activation of the enzyme in extracts of semi-anaerobically grown cells. At higher concentrations of GSSG the activation appeared to be instantaneous, so that an elevated activity was observed in the sample taken immediately after addition of the sulphur compound to the enzyme. A similar pattern of activation was observed with GSH except that there was no instantaneous activation, even at the highest concentrations of additive. The patterns of activation obtained with D-, DL- or L-cysteine and D-, DL-, *meso*-, L- or acid-treated cystine (prepared as described in the Materials and Methods section) were similar to that shown with GSSG except that equivalent effects were obtained at approximately 10-fold lower concentrations.

The instantaneous activations shown in Fig. 4 indicated that activation of the enzyme with sulphur compounds might also be demonstrated if the compounds were added directly to the enzyme–substrate mixture. GSSG, GSH and the cysteine and cystine preparations described above were all tested at a range of concentrations between 0.001 and 5mM. The specific activity obtained with the optimum concentration of each compound is given in Table 4. All compounds except GSH produced a 3–5-fold activation of the enzyme. The most effective activators were GSSG and acid-treated cystine, the cystine acting at a 10-fold lower concentration than GSSG. Acid-treated cystine appeared to be more soluble than untreated cystine at neutral pH and this may account for the high activations obtained with the acid-treated preparation. 2-Mercaptoethanol, 2-mercaptoethylamine, DL-methionine, DL-threonine, DL-homoserine, *S*-adenosylmethionine, L-cystathionine and lipoic acid were all without effect in this assay. The assay used for aminolaevulinate synthetase in this work requires enzymic formation of succinyl-CoA (3-carboxypropionyl-CoA) *in situ*. The activating effect of sulphur compounds was shown not to be due to alterations in the availability of this substrate. GSSG (1.0mM) produced no significant alteration in the succinate thiokinase activity (3500nmol of succinohydroxamate/h per mg of protein) of a crude low-activity preparation.

It was found that sulphur compounds only affected the low-activity form of aminolaevulinate synthetase. Thus, although GSSG increased the activity of crude low-activity enzyme from oxygenated cells and also that of the low-activity enzyme in extracts of semi-anaerobically grown cells immediately after cell disruption, none of the sulphur compounds shown in Table 4 activated crude high-activity enzyme (prepared as described in the Materials and Methods section).

Mechanism of the activation of low-activity enzyme with sulphur compounds

Marriott *et al.* (1970) found that spontaneous activation of aminolaevulinate synthetase could be totally inhibited by excluding air or adding NADH (0.5mM). We have found that the time-dependent

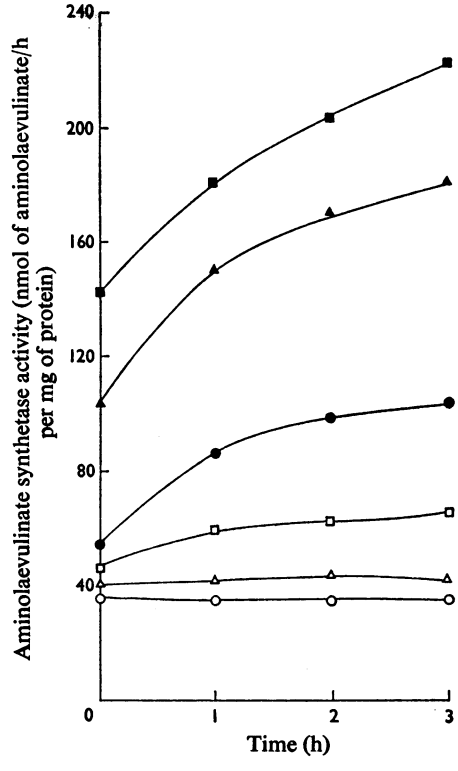


Fig. 4. *Effect of GSSG on crude low-activity aminolaevulinate synthetase*

To samples of crude low-activity enzyme (about 10mg of protein/ml) at 4°C, different amounts of GSSG were added to give concentrations between 0.05 and 5mM. At intervals, samples (100μl) were withdrawn into substrate mixture and when all samples had been taken the assay tubes (total volume 1.0ml) were incubated together at 37°C for 1h and the aminolaevulinate synthetase activity was determined. Concentrations of GSSG: ○, none; △, 0.05mM; □, 0.10mM; ●, 0.5mM; ▲, 1.0mM; ■, 5mM.

activation of low-activity enzyme with cysteine or cystine is also inhibited by excluding air or adding NADH. This finding indicates that the mechanism of the activation with sulphur compounds is similar to the spontaneous process and probably involves the oxidation of some endogenous factor via the electron-transport chain. To investigate the nature of any endogenous factors involved in the activation, a four-step purification procedure for low-activity enzyme was developed (see the Materials and Methods section). After each step the effect of acid-treated cystine and 'pellet factor' (prepared as described in the Materials and Methods section) on the activity of the preparation was tested (Table 5).

The crude enzyme extract could be activated up to 4-fold by the addition of cystine (0.08mM) alone. The pigment-free high-speed supernatant (Step 2), however, was virtually unaffected by cystine alone but was activated up to 4-fold when cystine was added together with the pellet factor. This pellet factor was a chromatophore-rich preparation, which exhibited no aminolaevulinate synthetase activity when assayed alone and did not activate the supernatant fraction when added in the absence of cystine. The effect of a range of concentrations of the pellet factor in the activation of the high-speed supernatant is shown in Fig. 5. The enzyme eluted from DEAE-cellulose (Step 3) also showed an absolute requirement for the pellet factor in the activation with cystine, and up to 3-fold activation was obtained under optimum conditions.

Further purification of the enzyme on Sephadex G-100 (Step 4) resulted in a preparation that was not activated by cystine either alone or in the presence of the pellet factor. A wide range of concentrations of both cystine and the pellet factor was tested and in no experiment was significant activation observed. Greater than 80% recoveries of activity were generally obtained from the column, so that it is unlikely that the loss of activability was due to an instability of the enzyme. The most reasonable explanation is that some endogenous factor, essential to the activation process, was inactivated or resolved from the enzyme on Sephadex G-100 chromatography.

Table 4. *Effect of sulphur compounds on the activity of crude low-activity aminolaevulinate synthetase*

Crude low-activity enzyme (about 0.8mg of protein) was added to tubes containing substrate mixture and sulphur compounds at a range of concentrations (total volume 1.0ml). Tubes were incubated at 37°C for 60min and the aminolaevulinate synthetase activity was determined. The table shows the specific activity obtained with the optimum concentration of each additive.

Additive	Optimum concentration (mM)	Aminolaevulinate synthetase activity (units/mg of protein)
None	—	34
GSH	0.5	40
GSSG	1.0	184
D-Cysteine	0.08	124
DL-Cysteine	0.10	126
L-Cysteine	0.10	102
D-Cystine	0.10	116
DL-Cystine	0.20	70
L-Cystine	0.10	96
Acid-treated cystine	0.08	186

Table 5. *Effect of acid-treated cystine and pellet factor on the activity of purified preparations of low-activity aminolaevulinate synthetase*

A preparation of aminolaevulinate synthetase from each of the four purification steps (see the Materials and Methods section) was added to tubes containing substrate mixture. Acid-treated cystine (to a concentration of 0.08mM), and pellet factor (10μl, prepared as described in the Materials and Methods section) were added as indicated. Incubation was at 37°C for 30–90min and the aminolaevulinate synthetase activity was determined.

Enzyme preparation / Additions ...	Aminolaevulinate synthetase activity (units/mg of protein)			
	None	Acid-treated cystine	Pellet factor	Acid-treated cystine and pellet factor
1. Crude extract	32	132	—	—
2. High-speed supernatant (pigment-free)	60	76	62	245
3. DEAE-cellulose eluate	76	99	82	224
4. Sephadex G-100 eluate	290	282	276	303

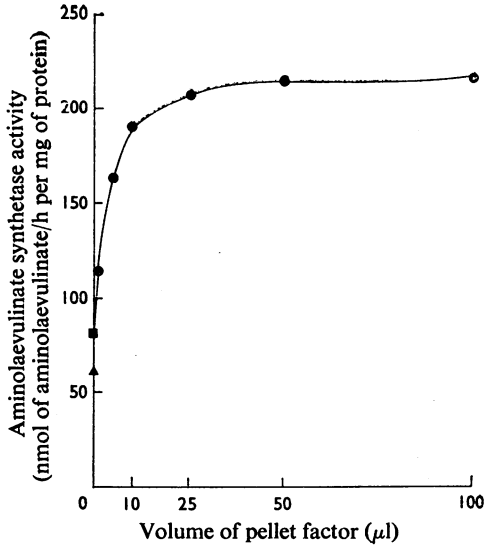


Fig. 5. *Requirement for pellet factor in the activation of low-activity aminolaevulinate synthetase activity with acid-treated cystine*

Aminolaevulinate synthetase extract (pigment-free supernatant, Step 2 of purification procedure; see the Materials and Methods section) was added to tubes containing substrate mixture and the following additions: ▲, none; ■, acid-treated cystine (0.08mM); ●, acid-treated cystine (0.08mM) and a range of concentrations of pellet factor (1–100μl) prepared as described in the Materials and Methods section. Incubation was at 37°C for 60min and the aminolaevulinate synthetase activity was determined.

Discussion

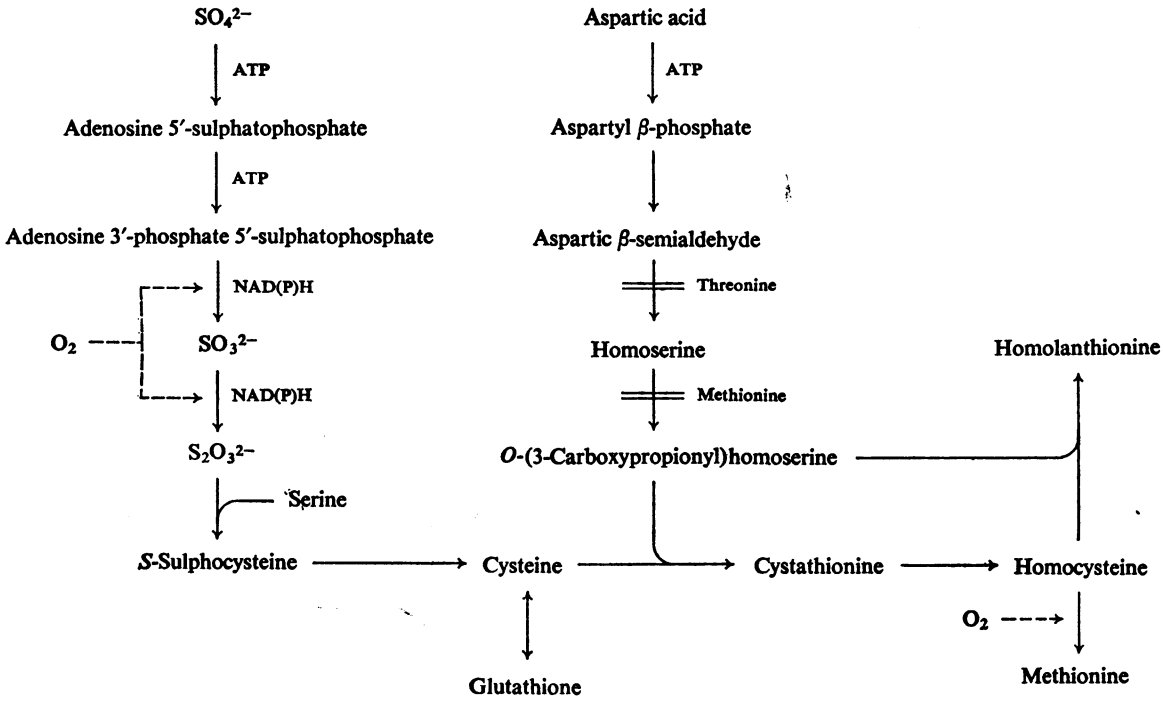
Effect of oxygenation on the sulphur metabolism of R. spheroides

The results in the present paper indicate that high oxygen partial pressure inhibits aminolaevulinate synthetase activity in *R. spheroides* by a mechanism directly involving the metabolism of sulphur compounds of low molecular weight. Rapid and marked changes in the concentrations of the major intracellular sulphur compounds of low molecular weight occur on oxygenation of a culture of *R. spheroides* growing semi-anaerobically in the light. Under a variety of experimental conditions the intracellular contents of GSH+GSSG and cysteine+cystine are directly correlated with the maximum aminolaevulinate synthetase activity observed in cell-free extracts. Losses of GSH+GSSG and aminolaevulinate synthetase activity on oxygenation depend on the ability of the cells to metabolize, as shown by the lack of effect of oxygen on cells suspended in phosphate buffer alone, and by its marked effect when malate or glutamate are present (Table 2). Losses of GSH+GSSG and enzyme activity do not occur when cells, able to metabolize, are oxygenated in the presence of a fresh supply of nutrients including sulphate. The mechanism of this effect is not clear, although it seems likely that in fresh medium the cells establish a rapid rate of synthesis of low-molecular-weight sulphur intermediates *de novo*, resulting in the maintenance of the content of GSH+GSSG even under oxygenation.

A proposed pathway for sulphur metabolism in *R. spheroides* is given in Scheme 1. The pathway is similar to that found in a variety of microorganisms (Meister, 1965; Kaplan & Flavin, 1966). Although none of the enzymes involved has been assayed in the present work, several of the intermediates have been identified in extracts and some of the individual steps have been demonstrated by other workers in cell-free extracts of *R. spheroides*. Peck (1962) found that crude extracts of this organism reduced adenosine 3′-phosphate 5′-sulphatophosphate to acid-volatile sulphur compounds, but no product was formed when adenosine 5′-sulphatophosphate was the substrate. The synthesis of methionine from homocysteine by crude extracts of *R. spheroides* has been observed by Cauthen *et al.* (1967), and the three enzymes involved in the conversion of aspartic acid into homoserine have been assayed in cell-free extracts of *R. spheroides* by Gibson *et al.* (1962).

Proposed sites for the effect of oxygen on the pathway are indicated by broken arrows. The protective effect of added thiosulphate and the lack of effect shown by sulphate, sulphite or sulphide (Table 3) indicates that oxygen inhibits the pathway at one or more steps in sulphate uptake or reduction and also that thiosulphate rather than sulphide is the reduced inorganic sulphur intermediate in *R. spheroides*. It is not unreasonable to suggest that the NAD(P)H-dependent reduction of sulphate might be sensitive to vigorous oxygenation of the culture. The reduction of adenosine 3′-phosphate 5′-sulphatophosphate by cell-free extracts of *R. spheroides* (Peck, 1962) could only be demonstrated under strictly anaerobic conditions. Inhibition of thiosulphate synthesis by oxygen would be expected to result in a rapid fall in the synthesis of cysteine *de novo* and it is probably this fall that initiates the observed degradation of glutathione.

The effect of oxygen on sulphur metabolism cannot, however, be confined to the pathway of sulphate reduction. The rapid conversion of almost all the glutathione into homolanthionine indicates



Scheme 1. *Postulated scheme for the metabolism of sulphur compounds of low molecular weight in R. spheroides*

The postulated sites of action for methionine and threonine are indicated by double lines. The possible sites for the effect of oxygen on the pathway are indicated by arrows. Some of the reactions indicated as unidirectional are probably reversible.

that oxygen also increases the rate of conversion of cysteine sulphur into homocysteine sulphur. Glutathione represents about 90% of the total low-molecular-weight amino sulphur compounds of *R. spheroides* grown semi-anaerobically, so that this change on oxygenation represents a major adjustment in the sulphur metabolism of the cell. The estimate obtained for GSH+GSSG in semi-anaerobically grown *R. spheroides* (about 5.5nmol/mg dry wt.) is rather higher than that reported by Knox (1960) for a variety of animal, plant and bacterial tissues, i.e. 0.15–3.0nmol/mg dry wt. A possible mechanism for the effect of oxygen is that it directly inhibits the conversion of homocysteine into methionine and thereby removes inhibitory control on the synthesis of *O*-(3-carboxypropionyl)-homoserine. Increased concentrations of *O*-(3-carboxypropionyl)homoserine would in turn result in an acceleration of the pathway from cysteine to homocysteine and thereby the observed accumulation of homolanthionine. The suggestion that oxygen directly inhibits methionine synthesis *in vivo* is consistent with the finding (Cauthen *et al.*, 1967) that methionine synthesis in extracts of *R. spheroides* requires the presence of $FADH_2$, NADH and a methyltetrahydrofolate derivative and can only be demonstrated under highly reducing conditions. The suggestion that methionine is a feed-back inhibitor of *O*-(3-carboxypropionyl)-homoserine synthesis in *R. spheroides* is consistent with the finding that added methionine prevented the conversion of glutathione into homolanthionine on oxygenation. Further, methionine is an inhibitor of *O*-(3-carboxypropionyl)homoserine synthetase in cell-free extracts of *Salmonella typhimurium* (Rowbury, 1965) and *Escherichia coli* (Lee *et al.*, 1966).

The protective effect of threonine (Table 3) can be explained by its inhibiting the formation of homoserine from aspartic β-semialdehyde, as shown in cell-free extracts of *R. spheroides* by Gibson *et al.* (1962*b*). The sites of action suggested for methionine and threonine (Scheme 1) are supported by the finding (Table 3) that the addition of homoserine

reversed the effect of threonine but not that of methionine. The effect of homocysteine (Table 3) may be explained by its inhibition of cystathionine synthesis as demonstrated in extracts of *S. typhimurium* by Kaplan & Flavin (1966).

The appearance of homolanthionine in oxygenated cultures is consistent with the suggestion that oxygen promotes the accumulation of homocysteine. Huang (1962) identified homolanthionine in the culture filtrate of an *E. coli* mutant blocked in the conversion of homocysteine into methionine. It has also been isolated from the urine of patients with homocystinuria (Perry *et al.*, 1966) and has been enzymically synthesized in extracts of rat and human liver (Tallan *et al.*, 1971).

Effect of oxygenation on the aminolaevulinate synthetase activity of R. spheroides

The results reported in the present paper provide an explanation for the effect of oxygenation of *R. spheroides* on the spontaneous activation of aminolaevulinate synthetase (Marriott, 1968). It is clear that for spontaneous activation of the enzyme to occur a high concentration of endogenous sulphur compounds of low molecular weight is required. Oxygenation of a culture growing semi-anaerobically in the light produces more than a 10-fold decrease in the amounts of GSH+GSSG and cysteine+cystine and thereby prevents spontaneous activation in the cell-free extract. The depletion of sulphur compounds and the loss of spontaneous activation are directly related changes, since activation of the enzyme can be brought about by the addition of any one of these sulphur compounds to the depleted extract. The importance of this finding, in relation to an understanding of the control of aminolaevulinate synthetase in *R. spheroides*, rests on the assumption that sulphur compounds also act in the cell to promote formation of the high-activity enzyme. This idea is supported by the finding that the activation of the enzyme with sulphur compounds is mediated by the action of a number of other endogenous factors.

One of these factors is confined to the particulate fraction of the cell and, considering the absolute requirement for air and the inhibitory effect of NADH in activation, it seems reasonable that this pellet factor provides an enzymic oxidizing potential, possibly involving electron transport. This would be consistent with the work reported by Marriott *et al.* (1970) in which electron transport mediated by oxygen or light was shown to be essential to the spontaneous activation process. Such an enzymically coupled oxidative system would also explain why either thiol or disulphide compounds promote activation of the enzyme. It can be visualized that a cyclic thiol–disulphide couple is involved, which is linked to the electron-transport chain. Supplying either the reduced or oxidized form of the couple would therefore be sufficient to initiate the cycle and promote activation of the enzyme.

From the work with partially purified enzyme, it is clear that at least one other endogenous factor is also required for activation of the enzyme. This factor appears to be associated with the enzyme throughout the first three steps of the purification procedure, but is resolved from the enzyme, or inactivated, by Sephadex G-100 filtration. The purification and properties of a low-molecular-weight compound from *R. spheroides* that activates aminolaevulinate synthetase in the absence of sulphur compounds, particulate fraction or air are described in the following paper (Neuberger *et al.*, 1973).

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