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Control of δ-Aminolaevulate Synthetase Activity in *Rhodopseudomonas spheroides*

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1. δ-Aminolaevulate synthetase from *Rhodopseudomonas spheroides* grown semi-anaerobically undergoes a spontaneous activation during the first hour after the disruption of cells when homogenates are stored at 4°. 2. After cultures of *R. spheroides* growing semi-anaerobically are oxygenated no activation of δ-aminolaevulate synthetase occurs in cell extracts. Cessation of activation in extracts is almost complete 10min. after oxygenation of cells has begun. 3. A heat-stable fraction of low molecular weight from semi-anaerobic cells reactivates δ-aminolaevulate synthetase in extracts of oxygenated cells and appears to contain a compound responsible for the spontaneous activation. 4. A heat-stable fraction of low molecular weight from oxygenated cells inhibits the spontaneous activation in extracts of semi-anaerobic cells. 5. The effect of oxygen on the rate of bacteriochlorophyll synthesis in *R. spheroides* may be mediated through alterations in the concentrations of a low-molecular-weight activator and inhibitor of δ-aminolaevulate synthetase.

When the photosynthetic bacterium *Rhodopseudomonas spheroides* is grown under air or oxygen, bacteriochlorophyll synthesis is repressed, and when it is transferred to conditions of low concentration of oxygen, synthesis begins (Lascelles, 1959; Gorchein, Neuberger & Tait, 1968). Synthesis of bacteriochlorophyll is accompanied by alterations in protein and lipid synthesis and by morphological changes involving the formation of chromatophores. One of the earliest changes observed in this adaptation is an increase in the activity of the first enzyme specific to porphyrin synthesis, ALA* synthetase. This suggests that this enzyme has an important role in the control of the branched pathway leading to the synthesis of haem, vitamin B_{12} and bacteriochlorophyll.

Burnham & Lascelles (1963) showed that haem is an inhibitor of ALA synthetase in cell-free extracts and Lascelles (1960) found that repression of ALA synthetase by haem occurs *in vivo*. It was found that when bacteriochlorophyll synthesis is inhibited by limitation of protein synthesis (Lessie & Sistrom, 1964) or by addition of ethionine or threonine (Gibson, Neuberger & Tait, 1962*b*) an increase in total porphyrin formation occurs, and this led to the suggestion that an intermediate in the bacteriochlorophyll branch of the pathway may also act as a feedback inhibitor of ALA synthetase.

When organisms adapted to anaerobic photosynthetic growth are transferred to an aerobic environment, synthesis of bacteriochlorophyll ceases immediately and ALA synthetase activity falls (Higuchi, Goto, Fujimoto, Namiki & Kikuchi, 1965; Gorchein *et al.* 1968). ALA synthetase in *R. spheroides* is labile *in vitro*, and it has been suggested that *in vivo* it is rapidly destroyed when synthesis is repressed, and that this inactivation is a factor in the control of bacteriochlorophyll synthesis (Higuchi *et al.* 1965). This hypothesis is supported by observations on ALA synthetase induced in liver mitochondria by drugs, where the enzyme activity falls after administration of inhibitors of protein synthesis (Marver, Collins, Tschudy & Rechcigl, 1966).

This paper reports the results of an investigation of ALA synthetase activity in extracts of *R. spheroides* grown semi-anaerobically and of the inactivation of this enzyme when semi-anaerobic cultures are oxygenated. Some aspects of this work have been discussed elsewhere (Marriott, 1968).

MATERIALS AND METHODS

Chemicals. CoA (90–95%) and ATP were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., pyridoxal phosphate and haemin were from British Drug Houses Ltd., Poole, Dorset, and 2-mercaptoethanol was from Koch–Light Laboratories Ltd., Colnbrook, Bucks. 2-Hydroxyethyl disulphide was prepared from 2-mercaptoethanol by dropwise addition of a calculated quantity of hydrogen peroxide solution at 4°. 2-Mercaptoethanol and hydrogen peroxide were standardized by titration against

* Abbreviation: ALA, δ-aminolaevulate.

I_2 and acidified KI respectively, and the 2-hydroxyethyl disulphide solution was shown to give no reaction with either of these reagents. Concentrations of these reagents are given as normalities with respect to reducing or oxidizing capacity.

Growth and harvesting of organisms. *Rhodopseudomonas spheroides* (N.C.I.B. 8253) cells were grown semi-anaerobically in the light as described by Gibson, Neuberger & Tait (1962*a*). Organisms were harvested in the late exponential phase of growth (0·4–0·6 mg. dry wt./ml.), suspended in buffer, washed and finally resuspended in buffer to a known concentration. The buffer used for suspending cells and for dilution of cell extracts was 0·05M-sodium phosphate, pH 7·0, unless stated otherwise. All timed samples from oxygenated cultures were diluted with a large volume of cold buffer before harvesting, to minimize metabolism between withdrawal of the cells and disruption.

Preparation of cell-free extracts. Cell suspensions were disrupted by ultrasonic treatment in an MSE ultrasonic power unit for 2 min. at 0°. Immediately afterwards extracts were centrifuged at 4° for 10 min. at 25000*g* to remove unbroken cells and debris and the supernatant was stored at the same temperature. When substrates or other compounds were mixed with the extract immediately after ultrasonic treatment, centrifugation was carried out after the additions.

Gel filtration. ALA synthetase activity is unstable on dialysis, and to remove compounds of low molecular weight from extracts, gel filtration at 4° on a column (1 cm. × 15 cm.) of coarse-grade Sephadex G-50 was used. This allowed very rapid gel filtration with no loss of ALA synthetase activity and caused dilution by a factor of less than two.

Determinations. The dry weight of organisms was calculated from a calibration curve by measurement of E_{680} of cell suspensions (Cohen-Bazire, Sistrom & Stanier, 1957). Protein concentration in extracts was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

Assay of ALA synthetase activity. The quantities of substrates and cofactors used and the determination of ALA generally followed the method of Lascelles (1960), except that glutathione was omitted from the substrate mixture. When exogenous succinyl-CoA was used as substrate this was prepared by the method of Simon & Shemin (1953) and 400 mμmoles were added instead of succinate, CoA, ATP and $MgCl_2$. Extracts, prepared as described above and containing 0·4–0·6 mg. of protein, were added to the whole substrate mixture, water at 4° was added to a total volume of 1 ml., and the mixture was stored at 4° before incubation at 37° for 30 min.

Assay of succinate thiokinase activity. The substrate mixture used was that for ALA synthetase except that glycine and pyridoxal phosphate were replaced by hydroxylamine (4M-hydroxylamine hydrochloride soln. adjusted to pH 7·8, 0·2 ml.). The incubation was carried out under the same conditions as those for ALA synthetase assay, and succinohydroxamate was determined by the method of Lipmann & Tuttle (1945).

Preparation of protein-free extracts from R. spheroides. Cell suspensions of *R. spheroides* (20 ml.) were prepared by harvesting, washing and suspending in water to a concentration of 100 mg. dry wt./ml. Semi-anaerobic organisms were extracted by stirring them at 4° with 10% (w/v) trichloroacetic acid (20 ml.) and after centrifugation the supernatant was neutralized with NaOH. To this neutralized supernatant ethanol was added to a final concentration of 90% (v/v), and the precipitate was dissolved in water (20 ml.). Semi-anaerobically grown organisms that had been oxygenated for 1 hr. before being harvested were extracted directly with ethanol (180 ml.). The supernatant was evaporated to dryness under reduced pressure, the residue was suspended in water (20 ml.), and pigmented material was removed by centrifugation at 105000*g* for 1 hr.

RESULTS

Spontaneous activation of ALA synthetase from R. spheroides

When cell suspensions of *R. spheroides* grown semi-anaerobically were disrupted and the extract was stored at 4°, the ALA synthetase activity showed a rapid increase in the first hour after disruption (Fig. 1). After this initial rise the activity was relatively constant for 2 hr., when it began to decrease. The time-dependence of ALA formation during incubation at 37° was measured for samples taken immediately after disruption and 1 hr. later. Although the rates of ALA formation differed by a factor of five they were both linear during the time of incubation (see Fig. 3), so that changes in ALA synthetase activity must have ceased when samples were diluted with substrate. After addition of substrate the same activity was obtained whether a sample was incubated at 37° immediately or was stored at 4° for a period of several hours before incubation. No detectable

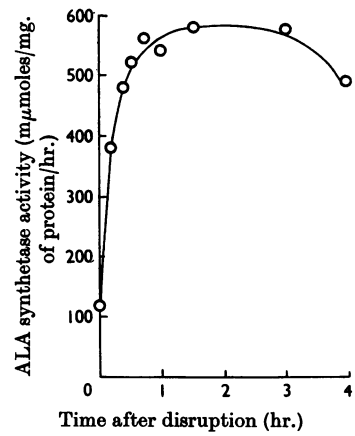

Fig. 1. Variation of ALA synthetase activity in *R. spheroides* extracts with time after disruption. *R. spheroides* cells (20 mg. dry wt./ml.) were disrupted and the supernatant after centrifugation at 25000*g* for 10 min. was stored at 4°. At the times stated portions (0·05 ml.) were removed and added to the substrate mixture for ALA synthetase and stored at 4° until 4 hr., when all samples were incubated at 37° for 30 min.

Table 1. *ALA synthetase activity from* R. spheroides *suspensions disrupted at various cell concentrations*

A suspension of *R. spheroides* grown semi-anaerobically was diluted to known concentrations; each sample was disrupted and portions were assayed for ALA synthetase immediately and 1 hr. later.

Concn. of cells (mg. dry wt./ml.)	Activity of ALA synthetase (mμmoles/mg. of protein/hr.)	
Time after disruption (hr.) ...	0	1
100	22	42
80	35	58
60	53	42
40	51	145
20	40	222
10	41	216
5	38	79
2·5	48	68

amount of ALA was formed at 4°, and samples for activation measurements were generally stored until all were available; they were then incubated simultaneously. In describing further work on this activation the activity immediately after disruption is referred to as the 'initial activity' and that 1 hr. after disruption as the 'maximum activity'.

Concentration-dependence of the activation. When cell suspensions of different concentrations were used, large activations were obtained only for initial concentrations between approx. 40 and 10 mg. dry wt./ml. (Table 1). If concentrated suspensions (100 mg. dry wt./ml.) were disrupted and subsequently diluted, activation began immediately after dilution (Fig. 2*a*). When cell suspensions (20 mg. dry wt./ml.) were disrupted and diluted immediately activation was partially or completely prevented (Fig. 2*b*). These results showed the rate and extent of activation to be dependent on concentration during activation but not on concentration during disruption. Once this activation had been discovered it was possible to obtain good reproducibility for the assay of ALA synthetase activity in crude cell homogenates. The activation was the same whether cells were disrupted by ultrasonic treatment or in a French pressure cell. It was necessary to obtain a high efficiency of disintegration for the activation to be reproducible.

Effect of succinate thiokinase on the assay of ALA synthetase. The assay used for ALA synthetase requires enzymic formation of succinyl-CoA *in situ*, and large changes in the rate of formation of this substrate might have caused the observed changes in the amount of ALA formed. When ALA syn-

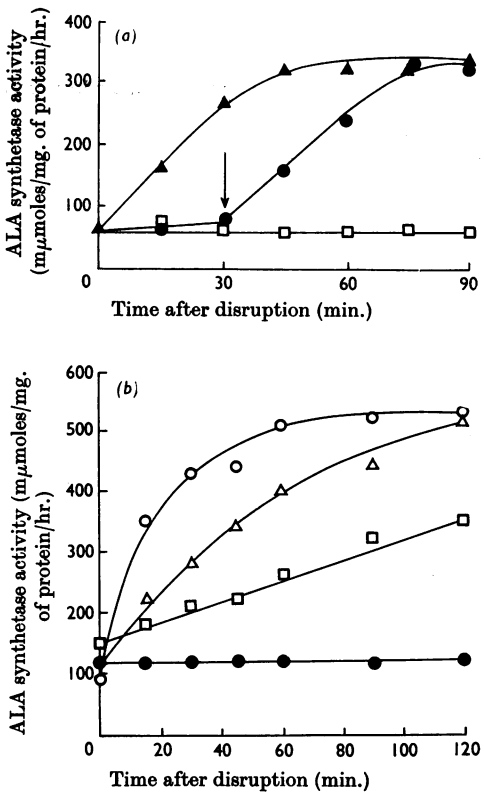

Fig. 2. Effect of dilution of *R. spheroides* extracts on ALA synthetase activity. (*a*) Suspensions of *R. spheroides* were disrupted and ALA synthetase activity was determined at the times stated. Concentration of suspensions (mg. dry wt. of bacteria/ml.): ▲, 25; □, 100; ●, 100; at the time shown by the arrow the extract was diluted fourfold. (*b*) *R. spheroides* cells (20 mg. dry wt./ml.) were disrupted, the extract was diluted immediately and ALA synthetase activities were determined at the times stated. Dilution factors: ○, ×1; △, ×2; □, ×4; ●, ×8.

Table 2. *Effect of succinate thiokinase on the assay of ALA synthetase*

R. spheroides cells (20 mg. dry wt./ml.) were disrupted and the enzyme assays carried out as described in the Materials and Methods section.

Assay	Reaction product (mμmoles/mg. of protein/hr.)	
Time between cell disruption and assay (hr.)	0	1
ALA synthetase (with a succinyl-CoA-generating system)	50	360
ALA synthetase (with added succinyl-CoA)	82	254
Succinate thiokinase	2530	2590

Table 3. *Effect of carboxylic acids on the activation of ALA synthetase*

R. spheroides cells (20mg. dry wt./ml., 1ml.) were disrupted and additions made (1·0M solutions, 0·01ml.). Samples for ALA synthetase measurement were taken after 1hr. The activity of extracts immediately after disruption was 110mμmoles of ALA/mg. of protein/hr.

Addition	Activity of ALA synthetase (mμmoles/mg. of protein/hr.)
None	610
Pyruvate	460
Glutamate	370
Succinate	270
β-Hydroxybutyrate	180
α-Oxoglutarate	160
cis-Aconitate	130
Citrate	115
Isocitrate	110

thetase was assayed with succinyl-CoA as substrate, activation was still observed (Table 2). Assay of ALA synthetase with added succinyl-CoA involved greater errors owing to the instability of the substrate, and all subsequent assays used enzymically generated succinyl-CoA. Confirmation that succinate thiokinase was not involved in the activation process was obtained when assay before and after ALA synthetase activation showed that its activity was unaffected (Table 2).

Optimum conditions for the activation. The activation was pH-dependent but had a broad optimum range. The maximum rate was at pH7·0; it was significantly lower at pH6·2 and 7·8. Alterations of ionic strength, by addition of sodium chloride up to 1·0M, did not affect the activation, nor did the use of tris buffer instead of phosphate. Addition of EDTA before or after disruption did not alter the rate of activation.

Inhibitors of the activation. Addition of components of the substrate mixture to extracts immediately after cell disruption showed that succinate completely inhibited the activation but none of the other components affected it. Other carboxylic acids were tested for their effects on activation and the ones that were inhibitory at 10mM are shown in Table 3. At concentrations of 10mM the following compounds did not affect the activation: acetate, propionate, glyoxylate, oxalate, malonate, fumarate, maleate, aspartate, malate, tartrate, oxaloacetate, glutarate and tricarballylate. When concentration-dependence was examined, citrate and α-oxoglutarate were found to be the most potent, being effective at 1mM but not at 0·1mM, whereas succinate and β-hydroxybutyrate were not effective at 1mM. When these compounds were added to fully activated extracts at a concentration of 10mM, β-hydroxybutyrate inhibited by 30% and the others by less than 10%. The effect of ALA on activation was measured by removing added ALA by gel filtration before assay; ALA was less effective than citrate but slightly more effective than succinate as an inhibitor of activation (Table 4).

Table 4. *Effect of ALA on the activation of ALA synthetase*

R. spheroides cells (1ml., 20mg. dry wt./ml.) were disrupted and additions made. After 1hr. low-molecular-weight compounds were removed by gel filtration and the specific activity of ALA synthetase was determined.

Addition	Concn. after addition (mM)	Activity of ALA synthetase (mμmoles/mg. of protein/hr.)
None	—	360
Citrate	10	65
ALA	10	65
ALA	1	145
ALA	0·1	250
ALA	0·01	350

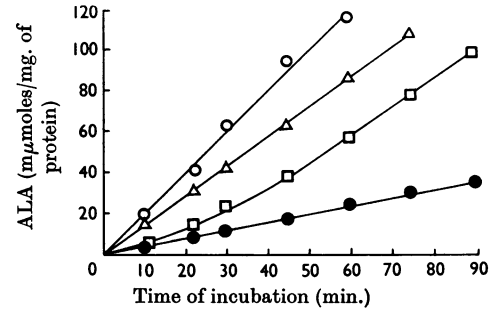

Fig. 3. Effect of 2-mercaptoethanol on the rate of formation of ALA during assay of ALA synthetase. *R. spheroides* cells (20mg. dry wt./ml., 1ml.) were disrupted, the substrate mixture for ALA synthetase (9ml.) was added, samples (1ml.) were withdrawn at the stated times during incubation at 37°, and ALA was determined. In Expt. A (○) the extract was stored at 4° for 2hr. before the addition of substrate; in Expts. B, C and D substrate was added immediately after disruption. In Expt. B (●) the mixture was incubated without further additions; in Expts. C and D 2-mercaptoethanol (1·0N, 0·1ml.) was added with the substrate. In Expt. C (□) the mixture was incubated immediately after addition of substrate and 2-mercaptoethanol; in Expt. D (△) it was stored at 4° for 2hr. before incubation.

Table 5. *ALA synthetase activity of* R. spheroides *extracts after treatment with 2-mercaptoethanol and 2-hydroxyethyl disulphide*

Identical treatments were carried out with extracts of *R. spheroides* (20mg. dry wt./ml., 2ml.) to which citrate (0·1M, 0·2ml.) was added immediately after disruption or 1hr. after disruption. Citrate was removed by gel filtration and 2-mercaptoethanol or 2-hydroxyethyl disulphide (0·1N, 0·2ml.) was added. After 30min. the additive was removed by gel filtration and then 2-mercaptoethanol or 2-hydroxyethyl disulphide (0·1N, 0·2ml.) was added again. After 30min. these were again removed by gel filtration. After each gel filtration the specific activity of ALA synthetase was determined.

Treatment	Activity of ALA synthetase (mμmoles/mg. of protein/hr.)	
Time between disruption and addition of citrate (hr.) ...	0	1
R. spheroides extract+citrate (I)	108	448
I+2-mercaptoethanol (II)	422	364
I+2-hydroxyethyl disulphide (III)	158	156
II+2-hydroxyethyl disulphide	93	108
III+2-mercaptoethanol	296	284

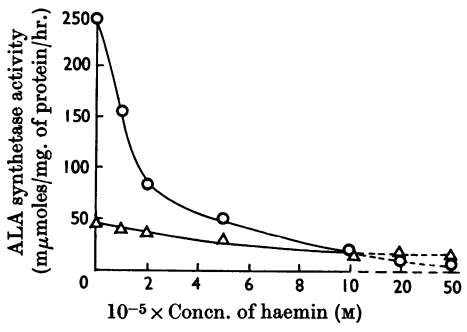

Fig. 4. Inhibition of ALA synthetase activity by haemin. *R. spheroides* cells (20mg. dry wt./ml.) were disrupted and added to the substrate mixture for ALA synthetase containing the stated concentration of haemin, and ALA synthetase activity was determined. Time between disruption and addition to the substrate–haemin mixture (hr.): △, 0; ○, 1.

Effect of 2-mercaptoethanol and 2-hydroxyethyl disulphide on ALA synthetase activity. It was considered possible that the initial low activity of ALA synthetase was an artifact caused during disruption, possibly by oxidation. However, the initial activity was not increased when nitrogen was bubbled through the cell suspension during ultrasonic treatment, nor did oxygenation of activated extracts cause any loss of activity. As no effect could be demonstrated by exclusion of oxygen, the effect of a reducing agent, 2-mercaptoethanol, was investigated (Fig. 3). The rate of formation of ALA before and after activation was linear (Expts. B and A). When substrate and 2-mercaptoethanol were added to an extract before activation and the mixture was stored at 4° for 2hr. before incubation at 37°, the rate of ALA formation was also linear (Expt. D), but when incubation was begun immediately after the addition the initial rate was slower and the rate of formation was non-linear (Expt. C). It appears that 2-mercaptoethanol stimulated the activity of extracts in which activation had not occurred, and that it did this at either 4° or 37°. When 2-mercaptoethanol was included during ultrasonic treatment, and immediately afterwards substrate was added, the rate of formation of ALA on immediate incubation at 37° was indistinguishable from that when 2-mercaptoethanol was added after ultrasonic treatment. Thus the presence of 2-mercaptoethanol during disruption did not alter the initial activity of ALA synthetase.

When extracts were treated with 2-mercaptoethanol before and after activation the activity of the former was increased and of the latter hardly altered (Table 5). When extracts were treated with 2-hydroxyethyl disulphide before and after activation the former was unaltered and the latter inhibited. The effect of one reagent was reversed by the other so that an extract inactivated by 2-hydroxyethyl disulphide was reactivated by 2-mercaptoethanol and one activated by 2-mercaptoethanol was inactivated by 2-hydroxyethyl disulphide.

Inhibition of ALA synthetase by haemin. Burnham & Lascelles (1963) showed that haemin was a potent inhibitor of ALA synthetase. When the effect of haemin on ALA synthetase activity before and after spontaneous activation was investigated, the activated form was markedly inhibited, in agreement with the results of Burnham & Lascelles (1963), but the low-activity form was almost unaffected by haemin (Fig. 4).

Isolation of an endogenous activator of ALA synthetase

When succinate and citrate were removed by gel filtration from extracts to which they had been added at disruption, no spontaneous increase in ALA synthetase activity occurred during storage at 4°. It was also found that activation could be prevented by gel filtration of a crude extract immediately after disruption. By adding back retarded column fractions it was possible to obtain increases in the activity of the excluded fractions. This activating capacity of the retarded fractions was stable to boiling. To isolate this heat-stable

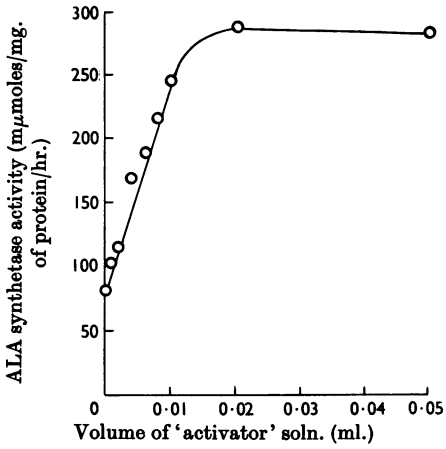

Fig. 5. Effect on ALA synthetase activity of the 'activator' prepared from *R. spheroides* grown semi-anaerobically. *R. spheroides* extract of low ALA synthetase activity was prepared by the addition of citrate immediately after the disruption of cells grown semi-anaerobically and gel filtration to remove all low-molecular-weight substances. 'Activator' was prepared as described in the Materials and Methods section. To the stated volume of 'activator' diluted with 0·05M-phosphate buffer, pH 7·0 (total vol. 0·2ml.), the extract (0·05ml.) was added and after 1hr. at 4° ALA synthetase activity was determined.

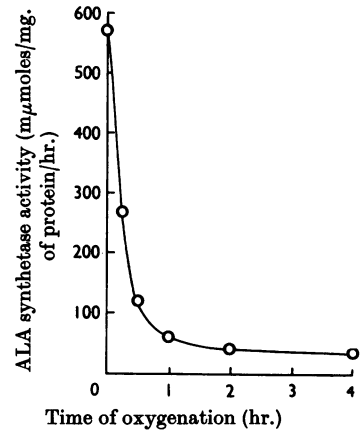

Fig. 6. Inactivation of ALA synthetase after oxygenation of *R. spheroides* growing semi-anaerobically. A culture of *R. spheroides* was oxygenated and samples were withdrawn at the times stated. Cells were harvested, washed, resuspended in buffer (20mg. dry wt. of bacteria/ml.) and ALA synthetase activity was determined 1hr. after disruption.

activator in bulk, a protein-free cell extract was prepared as described in the Materials and Methods section. To test this preparation a low-activity extract was prepared by addition of citrate at disruption and subsequent gel filtration. Addition of the extract caused a 3·5-fold increase in activity and the activity was proportional to the volume added, up to a limiting value (Fig. 5).

Thus the spontaneous activation of ALA synthetase is dependent on a heat-stable compound of low molecular weight occurring in *R. spheroides* grown semi-anaerobically.

Effect of oxygenation of R. spheroides *grown semi-anaerobically on ALA synthetase activity*

When semi-anaerobically grown *R. spheroides* was oxygenated and samples were withdrawn at intervals, the maximum ALA synthetase activity of extracts fell rapidly (Fig. 6). This is similar to changes observed by Lascelles (1960) and by Higuchi *et al.* (1965), with air, but the effect of oxygen is faster and greater (cf. Gorchein *et al.* 1968). When full activation curves were made for each sample, it was found that the first change caused by oxygen was suppression of the spontaneous activation of ALA synthetase, which had almost ceased after oxygenation for 10min. (Fig. 7).

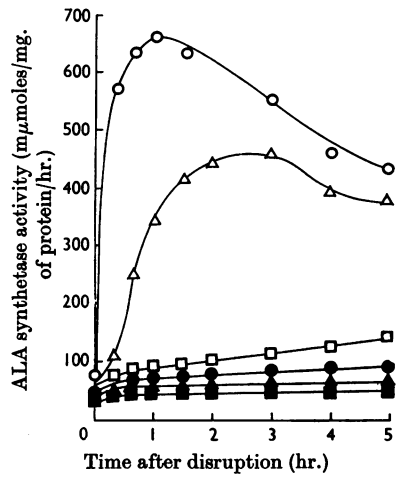

Fig. 7. Effect of oxygenation of *R. spheroides* growing semi-anaerobically on the activation of ALA synthetase in cell-free extracts. Conditions were as described for Fig. 6, but ALA synthetase activities were measured at the stated times after disruption. Time of oxygenation (min.): ○, 0; △, 5; □, 10; ●, 20; ▲, 30; ■, 60.

The initial activity at disruption also fell significantly and was decreased by about 50% after 1hr. of oxygenation.

Activation of ALA synthetase in extracts from oxygenated R. spheroides. Rapid turnover of ALA

Table 6. *Activation of ALA synthetase in extracts of oxygenated* R. spheroides

R. spheroides cultures were oxygenated, harvested, washed and resuspended in buffer (100mg. dry wt. of bacteria/ml.). After disruption portions (0·05ml.) were added to 0·05M-phosphate buffer, pH7·0, containing 'activator' from semi-anaerobic *R. spheroides* or citrate (total vol. 0·2ml.). After 1hr. portions (0·05ml.) were taken for ALA synthetase determination.

Addition	Volume (ml.)	Activity of ALA synthetase (mμmoles/mg. of protein/hr.)		
Time of oxygenation (min.) ...		0	20	60
None	—	488	117	56
0·1M-Citrate	0·02	97	65	39
'Activator'	0·01	—	181	80
'Activator'	0·02	—	306	131
'Activator'	0·04	—	362	194
'Activator'	0·10	—	400	221
'Activator'	0·20	—	—	288

synthetase has been suggested to account for the observed decreases in activity when cultures are aerated (Higuchi *et al.* 1965). As the initial effect of oxygen was to inhibit activation it seemed possible that the enzyme might be activated by addition of the 'activator' from semi-anaerobic cells. When the protein-free extract from anaerobic cells was added to extracts from cells that had been oxygenated, increases in activity occurred and the degree of activation, calculated as the ratio of maximum activity to initial activity, was similar to that obtained spontaneously from semi-anaerobic cells (Table 6). A similarly prepared extract from oxygenated cells did not increase the activity of extracts; thus the activating component for ALA synthetase appeared to be lost when cells were oxygenated. The initial activity showed a decrease of about 60% after oxygenation for 1hr., and, as the degree of activation was not significantly higher for this sample than for the control sample, this decrease was not reversed by the activator and it may be caused by turnover.

Isolation of an endogenous inhibitor of ALA synthetase activation

Inhibition of ALA synthetase activation by whole extracts of oxygenated R. spheroides. Experiments were carried out by preparing extracts from oxygenated cells and adding these to semi-anaerobic cells immediately after disruption. The extract from oxygenated cells partially inhibited activation at a ratio of oxygenated to semi-anaerobic cell extract of 1:5 and completely prevented it at 1:1. If the extracts were mixed when both had been stored for 1hr. after disruption, the activity of the mixture was the sum of its components; thus the oxygenated extract interfered only with the activation process.

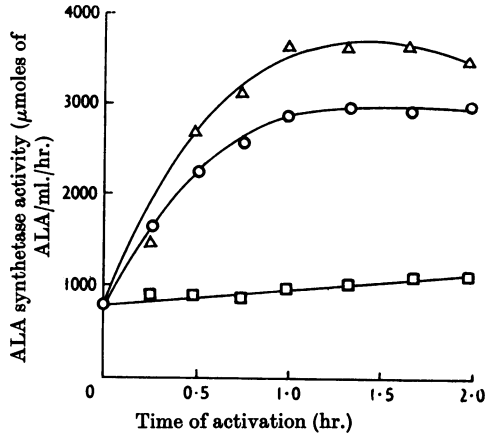

Fig. 8. Activation of ALA synthetase in mixtures of extracts from semi-anaerobic and oxygenated *R. spheroides*. A culture of *R. spheroides* was oxygenated for 1hr., a cell-free extract was prepared (I), and part of it was treated by gel filtration (II). Both solutions were diluted to about the same protein concentration (10mg./ml.) and ALA synthetase activity (100mμmoles of ALA/ml./hr.). Semi-anaerobic *R. spheroides* cells (20mg. dry wt./ml.) were disrupted, diluted with the extracts from oxygenated cells or buffer and ALA synthetase activities were observed at the times stated. Additions to the extract from semi-anaerobic cells (0·5ml.): ○, buffer (0·5ml.); □, I (0·5ml.); △, II (0·5ml.).

When the oxygenated cell extract was passed through a column of Bio-Gel P2 and then added to semi-anaerobic cells immediately after their disruption it no longer interfered with activation and the final activity of the mixture was greater than the sum of its components (Fig. 8). This increase is probably due to activator in the semi-

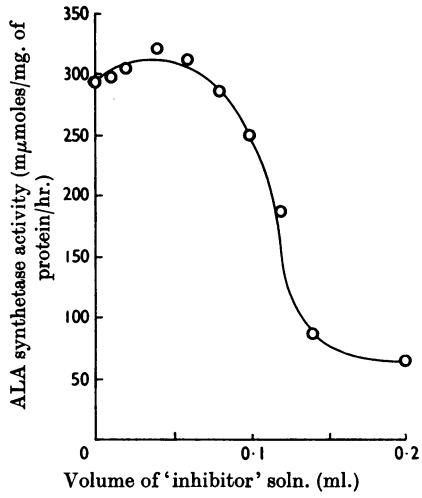

Fig. 9. Effect on the spontaneous activation of ALA synthetase of the 'inhibitor' from oxygenated *R. spheroides*. *R. spheroides* cells (100mg. dry wt./ml.) were disrupted and portions (0·05ml.) were added immediately to 'inhibitor' (see the Materials and Methods section) diluted with buffer (total vol. 0·2ml.). After 1hr. at 4°, portions (0·05ml.) were withdrawn for assay of ALA synthetase activity. ALA synthetase activity immediately after disruption was 58mμmoles of ALA/mg. of protein/hr.

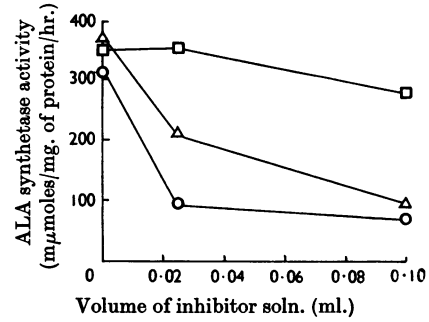

Fig. 10. Antagonistic effect of 'activator' and 'inhibitor' on the spontaneous activation of ALA synthetase. The conditions were the same as for Fig. 9, but 'activator' was included with the 'inhibitor' and buffer. Volume of 'activator' (ml.): ○, 0; △, 0·02; □, 0·10.

anaerobic extract acting on the low-activity ALA synthetase in the added extract. The results indicate the presence of an inhibitor of ALA synthetase activation in extracts of oxygenated cells.

Inhibition of ALA synthetase activation by an ethanol-soluble fraction from oxygenated R. spheroides. A protein-free extract was prepared from oxygenated cells as described in the Materials and Methods section. This solution was an effective inhibitor of ALA synthetase activation (Fig. 9). Up to a critical volume (0·1ml.) the activation was not significantly affected, but twice this volume almost completely inhibited activation, showing that there was a range over which small changes in inhibitor concentration caused a marked effect on the activation.

Antagonistic effects of protein-free extracts from semi-anaerobic and oxygenated R. spheroides. When protein-free extracts from semi-anaerobic and oxygenated cells were mixed the effect of the latter on spontaneous activation was partially reversed (Fig. 10). When low amounts of 'activator' were mixed with 'inhibitor' activation was partially prevented, and when larger amounts of 'activator' were used the effect of the 'inhibitor' was very small.

DISCUSSION

The presence of an inhibitor of ALA synthetase in aerobically grown *R. spheroides* was reported by Kikuchi, Kumar, Talmage & Shemin (1958), but without experimental details. The same authors found increases in total activity during purification of ALA synthetase, and similar increases have been observed in this Laboratory (Matthew, 1962). However, Lascelles (1959) found no evidence for an inhibitor in mixing experiments with extracts from semi-anaerobic and aerobic cells. The discovery of a spontaneous activation of ALA synthetase in extracts of semi-anaerobic cells makes it possible to account for these discrepancies. Extracts of oxygenated cells prevent activation if added before it occurs but do not reverse it if added afterwards. Consequently the results of mixing experiments are dependent on the timing of the preparation and mixing of extracts. Activation during purification would occur if the initial extracts were of high concentration and were diluted during fractionation (cf. Fig. 2*a*).

Investigations of the activation were confined to crude cell homogenates and this prevented any attempts to study the mechanism. The results presented on concentration-dependence and the effect of thiol compounds are relevant to reproduction of the activation rather than to understanding the mechanism. The effect of mercaptoethanol is particularly important as this has frequently been included in the assay system for ALA synthetase (Burnham & Lascelles, 1963). Mercaptoethanol increased the activity from extracts of semi-anaerobic cells when spontaneous activation was incomplete (Fig. 3) and also increased the activity of extracts from oxygenated cells. Thus, although

it stimulates the enzyme under some conditions, its inclusion in the assay system obscures alterations in activity.

At least two compounds, an activator and an inhibitor, appear to participate in the control of ALA synthetase activity in *R. spheroides* and to account for the activity changes caused by mixing whole extracts with each other or with deproteinized fractions. Both compounds are heat-stable and apparently of low molecular weight, but the activator is insoluble in 90% (v/v) ethanol whereas the inhibitor is soluble. From experiments with mixtures of these two components it is clear that the final activity of extracts is determined by the ratio of their concentrations (Fig. 10). The compounds may interact directly with ALA synthetase or may initiate reactions in the extract that lead to changes in ALA synthetase. Spontaneous activation of ALA synthetase in cell extracts is almost completely suppressed 10min. after commencement of oxygenation of a semi-anaerobic culture (Fig. 7), and this is probably caused by a decrease in the ratio of activator to inhibitor. This change is so rapid that it may well account for the cessation of bacteriochlorophyll synthesis in response to increase of oxygen concentration.

Since it is possible to activate ALA synthetase *in vitro* in the extracts with low specific activity obtained after oxygenation of semi-anaerobically grown *R. spheroides*, it is apparent that the inactivation *in vivo* is not caused by protein degradation but by a modification of the enzyme to give a low-activity form. Rapid inactivation of an induced enzyme after transfer of micro-organisms to a non-inducing environment has been observed with several other enzymes. Lysine decarboxylase of *Escherichia coli* was induced by lysine, and dialysed extracts required no exogenous coenzyme, but when the organisms were transferred to lysine-free medium extracts were apparently inactive unless pyridoxal phosphate was added (Mandelstam, 1954). Thus pyridoxal phosphate, essential to this enzyme, was non-diffusible but was rapidly removed *in vivo* after a change of environment. Ornithine transcarbamoylase of *Saccharomyces cerevisiae* was inactivated when arginine was added to cultures, but was reactivated by heating whole cells or extracts (Becket & Wiame, 1965). Fructose 1,6-diphosphate 1-phosphohydrolase of *S. cerevisiae* was inactivated when glucose was added to cultures and partially reactivated by heating extracts (Harris & Ferguson, 1967). In *Bacillus licheniformis*, threonine dehydratase was inactivated when cultures became glucose-deficient, but the quantity of enzyme, measured immunologically, remained constant during the change (Leitzmann & Bernlohr, 1968). As the inactivation was not prevented by inhibition of protein synthesis, the authors concluded that it might be caused by a metabolite that acted as an enzyme activator, which accumulated during growth on glucose but was depleted during glucose deficiency.

In all these cases enzyme activity varies with the growth conditions of cells before harvesting, but the quantity of enzyme protein can be demonstrated to be unaltered either by reactivation or by immunoassay. Enzyme activity may be altered by formation of inhibitors or activators that are firmly bound and are not removed by dialysis. These changes contrast with most mechanisms so far investigated for fine control of enzyme activity, where regulation usually occurs through a freely reversible interaction. In these cases the activity of enzymes in dialysed extracts is independent of the endogenous concentration of effector compound. It is possible that regulation of adaptive enzymes by low-molecular-weight compounds that can be firmly bound but are metabolically labile is a widespread mechanism in the adaptation of micro-organisms to changes in environment.

This mechanism contrasts with that operating in control of ALA synthetase in liver mitochondria. In studies on experimental porphyria in rats it was shown that the kinetics of inactivation of induced ALA synthetase after addition of protein-synthesis inhibitors were consistent with turnover of this enzyme with a half-life of 68–73min. (Marver *et al.* 1966). However, in liver, ALA synthetase appears to be the sole point of regulation of porphyrin synthesis as its activity is always proportional to the rate of porphyrin excretion (Granick, 1966). This situation is more simple than that in *R. spheroides* with its branched pathway of porphyrin synthesis and multiple controls (Lascelles, 1968).

This work raises a number of questions about the mechanism of integration and regulation of porphyrin synthesis in *R. spheroides*.

(1) What are the structures of the activator and inhibitor and what is the nature of their interaction with ALA synthetase?

(2) Why is the initial activity of ALA synthetase low in extracts of semi-anaerobic *R. spheroides* that synthesize bacteriochlorophyll?

(3) Why does the low-molecular-weight component from oxygenated cells prevent activation of ALA synthetase but not reverse it *in vitro*?

Purification of ALA synthetase is necessary for further investigations, but owing to the lability of the enzyme it has not been possible to obtain more than an incomplete purification (cf. Burnham & Lascelles, 1963).

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