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

THE PURIFICATION AND PROPERTIES OF AN ENDOGENOUS ACTIVATOR OF THE ENZYME

By ALBERT NEUBERGER, JOHN D. SANDY and GEORGE H. TAIT

*Department of Chemical Pathology, St. Mary's Hospital Medical School,
London W.2, U.K.*

(Received 16 March 1973)

1. A low-molecular-weight activator of 5-aminolaevulinate synthetase was detected in extracts of *Rhodopseudomonas spheroides*. The compound activates the enzyme extracted from oxygenated semi-anaerobically grown organisms by a factor of 6-8. 2. The activator was extensively purified, but owing to the exceedingly small amounts that could be extracted in the active form its structure was not determined. 3. The activator contains an acetylable amino group; it is more stable at acid than at alkaline pH values; it is stable to treatment with I_2 -KI or potassium ferricyanide, but irreversibly inactivated by $Na_2S_2O_4$ or $NaBH_4$. 4. The chromatographic, electrophoretic, chemical and stability properties of the activator are similar to those of pteridines; purified activator preparations contain pteridines, as shown by their fluorescence spectrum. This does not, however, constitute an identification of the activator. 5. The activator enhances the activity of crude and partially purified enzyme and does not appear to require other endogenous factors or a supply of air to produce activation. Activation of the purified enzyme, however, requires the presence of either pyridoxal phosphate or sodium succinate. In the absence of both these factors the activator produces a time- and temperature-dependent decay of activity.

The 5-aminolaevulinate synthetase of *R. spheroides* can exist in low- and high-activity forms *in vitro*. In the preceding paper (Neuberger *et al.*, 1973) we described the activation of the low-activity form in crude extracts by GSH, GSSG, cysteine or cystine. This activation was dependent on a supply of air, a particulate fraction and at least one other endogenous factor, which appeared to be resolved from the enzyme or inactivated by filtration of the partially purified enzyme on Sephadex G-100.

In the present paper we describe the purification and properties of a low-molecular-weight activator of aminolaevulinate synthetase in *R. spheroides*, which is not GSH, GSSG, cysteine or cystine and which appears to be active at μM concentrations. Evidence has been obtained that the compound produces activation by direct interaction with aminolaevulinate synthetase.

Materials and Methods

Chemicals

Pterin, biopterin, 6-carboxypterin, Dowex ion-exchange resins and Florisil (200-300 U.S. mesh) were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.; 6-methylpterin, 6-hydroxymethylpterin, 6,7-dimethylpterin

and 5,6,7,8-tetrahydropterin were all kindly supplied by Dr. H. S. Forrest, University of Texas, Austin, Tex., U.S.A.; dansyl chloride was from BDH Chemicals Ltd., Poole, Dorset, U.K.; [3H]acetic anhydride was from The Radiochemical Centre, Amersham, Bucks., U.K. Other chemicals were obtained from the sources described by Neuberger *et al.* (1973).

Methods

The growth and harvesting of organisms, preparation of cell-free extracts, determination of dry weight and protein, assay of enzymic activities, procedure for high-voltage electrophoresis at pH 2.0, the preparation of crude 'low'-activity and crude 'high'-activity enzyme, the four-step purification of low-activity aminolaevulinate synthetase and the preparation of 'pellet factor' are all described in the preceding paper (Neuberger *et al.*, 1973). Ninhydrin-positive compounds were determined by the method of Rosen (1957). Phosphocellulose was prepared as described by Peterson & Sober (1956) and was recycled with ammonium formate and formic acid as described by Rembold & Buschmann (1962). Phosphocellulose was mixed with 10% (w/w) of untreated cellulose to improve its flow-rate properties.

Acetylation of low-molecular-weight extracts

Freeze-dried preparations were dissolved in 1 ml of a sodium acetate solution (50%, w/v, in water) and, over a period of 1 h at 4°C, 0.20 ml of acetic anhydride was added with mixing. After the addition of 2 ml of water and standing at 4°C for a further 2 h, the solution was freeze-dried.

Dansylation of low-molecular-weight extracts

Freeze-dried preparations were dissolved in 25 μ l of NaHCO₃ (0.50M) and 25 μ l of dansyl chloride reagent (5 mg/ml in acetone); the mixture was incubated for 3 h at room temperature and then evaporated to dryness under vacuum.

Results

Assay of activator activity

The activator activity of low-molecular-weight extracts was determined by the addition of the extract, at a range of concentrations, to the assay mixture for crude low-activity enzyme (see the Materials and Methods section for details of enzyme preparation). Activator activity is therefore expressed on all figures in terms of aminolaevulinate synthetase activity.

Fig. 1 shows the assay of a crude activator extract prepared as described below. At the optimum concentration of added activator, a 5–6-fold activation was obtained, whereas at concentrations of activator above the optimum slight inhibition of the assay was observed. Activator extracts gave no colour when assayed for aminolaevulinate as described in the Materials and Methods section. Small amounts of aminolaevulinate, about 15% of the normal amount, were formed when crude extract was assayed in the absence of any one of the substrates. The addition of activator to assays done under these conditions did not increase the yield of aminolaevulinate.

In the assay system used, succinyl-CoA (3-carboxypropionyl-CoA), one of the substrates for aminolaevulinate synthetase, was formed *in situ* by the action of endogenous succinate thiokinase. The crude activator produced no significant effect on succinate thiokinase in a crude low-activity enzyme preparation. The effect of endogenous activator on the assay (Fig. 1) may thus be due to a specific activation of aminolaevulinate synthetase.

Purification of activator from *R. spheroides*

R. spheroides was grown semi-anaerobically in the light to about 0.5 mg dry wt./ml. After harvesting and washing, organisms were suspended in 0.05M-phosphate buffer, pH 7.0, to about 50 mg dry wt./ml and 50 ml batches were dialysed in Visking tubing

(18/32) against water for 20–24 h at 4°C. For efficient dialysis, the bags were placed in glass tubes (3 cm $\times$ 150 cm) that held about 1 litre of water and were stoppered at both ends. The tubes were placed on an electrically driven rocking platform. The recovery of activity was not significantly improved by sonication of the cells before dialysis. The combined difusate from 150 mg dry wt. of cells was applied to a column (2.5 cm $\times$ 5 cm) of Dowex 50 (X2; H⁺ form; 200–400 mesh) at about 2.5 ml/min. The column was eluted with water (50 ml) and 2M-NH₃; the first 20 ml of NH₃ off the column was collected and freeze-dried and the extract was dissolved in 10 ml of water to give crude activator.

Crude activator was fractionated in 25 ml batches on Dowex 50 by stepwise elution with HCl (Fig. 2). Some activity was eluted with 2M-HCl, but most of the activity was eluted with 4M-HCl. Most of the ninhydrin-positive material, including cystine, was eluted before the application of 4M-HCl, so that a marked purification of the activator was always obtained by this procedure. The recovery of activator activity from this column was generally greater than 100%, probably because the activator was resolved from compounds that inhibit the activation process.

The activity recovered from Dowex 50 with 4M-HCl was then chromatographed on Bio-Gel P-2 (Fig. 3). The combined active fractions from this column were further fractionated by chromatography on

Fig. 1. Assay of crude activator

To the stated volume of crude activator were added aminolaevulinate synthetase substrates (0.25 ml) and water (total volume, 0.9 ml). The mixture was incubated at 37°C for 3 min before the addition of crude low-activity enzyme (0.10 ml, about 0.8 mg of protein). Incubation was at 37°C for 60 min and the aminolaevulinate synthetase activity was determined.

Fig. 2. *Chromatography of activator on Dowex 50*

Crude activator (25ml) was diluted to 50ml in water and applied to a column (0.9cm × 10.0cm) of Dowex 50 (X2; H⁺ form; 200–400 mesh) at 36 ml/h. The column was then eluted as follows: (i) water (25ml), (ii) 0.5M-HCl (50ml), (iii) 1.0M-HCl (50ml), (iv) 2.0M-HCl (50ml), (v) 4.0M-HCl (60ml). Fractions (about 3ml) were dried under vacuum in a desiccator containing NaOH. Residues were dissolved in 1ml of water and assayed for activator activity (●) and ninhydrin-positive compounds (▲).

Fig. 3. *Chromatography of activator on Bio-Gel P-2*

The combined active fractions from Dowex 50 chromatography (fractions 62–69, Fig. 2) were freeze-dried, the extract was dissolved in 0.5ml of water and was applied to a column (2.0cm × 87.0cm) of Bio-Gel P-2 (100–200 mesh) previously equilibrated in 0.18M-acetic acid. The column was eluted with 0.18M-acetic acid at about 12 ml/h and 11 ml fractions were collected. Fractions were freeze-dried, the extracts dissolved in 1 ml of water and assayed for activator activity (●) and ninhydrin-positive compounds (▲). The recovery of activator activity was generally 65–85%.

Fig. 4. Chromatography of activator on phosphocellulose

The combined active fractions from Bio-Gel P-2 chromatography (fractions 22–26, Fig. 3) were freeze-dried, the extract was dissolved in 1 ml of water and was applied to a column (0.9 cm × 20.0 cm) of phosphocellulose, previously packed in water under an N_2 pressure of 51 lb/in² (35 kPa). The column was eluted with water at about 35 ml/h and 3 ml fractions were collected. Each fraction was assayed for activator activity (●) and ninhydrin-positive compounds. Only the activator activity is shown, since fractions gave no significant colour when tested with ninhydrin.

phosphocellulose (Fig. 4). At this stage two peaks of activator activity were obtained, which were labelled PI and PII. The recovery of activity off phosphocellulose varied between 35 and 65% and approximately equal amounts of activity were eluted under peaks PI and PII. Peaks PI and PII were reproducibly eluted at 1.5 times and 3.7 times the bed volume of the phosphocellulose column. Unless otherwise stated, activator recovered from phosphocellulose was used in the study of the properties of the activator and in the experiments described below on the interaction of activator and aminolaevulinate synthetase.

Other fractionation properties of purified activator

On high-voltage electrophoresis at pH 2.0 (Fig. 5) the activator migrated with a mobility relative to alanine of +0.48, and there was no significant difference in mobility for activator prepared from peaks PI or PII from phosphocellulose. The recovery of activity from high-voltage electrophoresis was very variable. Although in most experiments 40–60% recovery was obtained, some fractionations resulted in a total loss of activity. The cause for this variation

is uncertain, but recovery was always greater than 80% if all the steps in the procedure, except the application of voltage, were carried out exactly as described in the legend to Fig. 5.

Activator had a very low solubility in organic solvents. It could not be extracted into diethyl ether or ethyl acetate from aqueous solutions at neutral pH values, in 0.01 M-HCl or in 0.5 M-NaCl. Recovery of activity in the aqueous phase was generally 60–80%.

Activator was bound to Dowex 1 (X2; acetate form; 200–400 mesh) in water at pH 5.5 and could be recovered in good yield by elution with 0.5 M-acetic acid. Similarly, the activator was bound to the chloride form of this resin in water at pH 5.5 and could be recovered by elution of the column with 1 mM-HCl.

The activator was strongly absorbed to a column (1 cm × 7 cm) of Florisil (200–300 mesh) in 0.35 M-acetic acid. Little or no activity was recovered by elution with aq. 50% (v/v) acetone.

The apparent molecular weight of the activator was determined by gel filtration on columns of Bio-Gel P-2 (100–200 mesh; 2.0 cm × 87 cm) and Sephadex

Fig. 5. Preparative high-voltage electrophoresis of activator

The combined active fractions from phosphocellulose [either fractions 6–10 (PI) or fractions 18–22 (PII), Fig. 4] were freeze-dried and the extract was dissolved in 250 μ l of water. This preparation was applied under a stream of cold air across an 8 cm strip at one end of a sheet (22 cm $\times$ 56 cm) of Whatman 3MM paper which had previously been washed in 1 M-HCl and water. Electrophoresis was performed in 20% (v/v) formic acid–7.8% (v/v) acetic acid, pH 2.0, at 3 kV and 65 mA for 30 min. The paper was dried at room temperature and was cut into 0.5 cm strips. Compounds were recovered from the paper by placing one end of each strip between two microscope slides in a beaker of water, cutting the other end into a point and allowing the water to soak through the paper into a collecting vessel. Eluates were freeze-dried, the extracts were dissolved in 1 ml of water and assayed for activator activity.

G-10 (particle size 40–120 μ m; 0.9 cm $\times$ 115 cm). Elution was performed with 0.18 M-acetic acid and columns were calibrated with GSSG, GSH, cystine and cysteine. On both columns the elution volume for the activator indicated a molecular weight between 100 and 600.

Stability of the activator

Crude, partially purified and purified preparations of activator were stable when treated with 6 M-HCl at 110°C for 16 h under anaerobic conditions. Total loss of activity was always observed, however, if the hydrolysis was carried out in the presence of air.

Purified activator was completely stable in 0.2 M-NaOH for 2 h at room temperature but was destroyed after 10 min at 100°C.

Activator was destroyed by treatment with per-formic acid (formic acid–H₂O₂, 9:1, v/v) for 1 h at room temperature, but was unaffected by I₂–KI

(0.01 M-I₂, 30 min, room temperature) or potassium ferricyanide (0.01 M, 30 min, room temperature). The activator was inactivated by Na₂S₂O₄ (0.05 M, 30 min, room temperature) or NaBH₄ (0.1 M, 20 min, room temperature), and activity could not be recovered by subsequent treatment with I₂–KI (0.05 M-I₂, room temperature).

Evidence for an amino group in the activator

Purified activator was acetylated under the conditions described in the Materials and Methods section. The acetylated preparation was then dissolved in water and applied to a column (1 cm $\times$ 7 cm) of Dowex 50 (X2; H⁺ form; 200–400 mesh) and the column was eluted with water (40 ml) and then with 2 M-NH₃ (40 ml). All of the activity that could be recovered (about 65% of the total before acetylation) was present in the water eluate. This is in contrast with the properties of untreated activator on Dowex 50 (Fig. 2), and indicated the formation of an *N*-acetyl derivative of the activator.

Acetylation also markedly altered the migration of the activator on electrophoresis at pH 2.0. The product had a mobility relative to alanine of less than 0.10 as compared with a value of 0.48 for the untreated activator. The acetylated activator was bound very strongly to Dowex 1 and could not be recovered by elution with 0.5 M-acetic acid. Up to 30% of the activity in acetylated preparations could be extracted into diethyl ether or ethyl acetate at neutral pH. It was not possible, however, to use acetylation as a purification technique, since the acetylated compound was much less stable than the untreated activator to fractionation.

Acetylated activator was heated at 100°C for 3 h under anaerobic conditions in 2 M-HCl and also treated with 2 M-NH₃ at 4°C for 2 h. The sample treated with mild alkali retained the original fractionation properties of the acetylated compound on Dowex 50 and on high-voltage electrophoresis, whereas that treated with acid now exhibited the fractionation properties of untreated activator. These results further support the suggestion that the activator contains an amino group.

Purity of activator preparations

Since the biological activity of activator preparations was the only method which could be used to determine the concentration of activator present, it was not possible to assess accurately the degree of purification obtained. Purified activator extracts from phosphocellulose (Fig. 4) were ninhydrin negative. The ninhydrin method employed (Rosen, 1957) could detect 5–10 nmol of α -amino acid, so that if the activator contained a ninhydrin-reactive group it must have been effective at less than 2.0 μ M in the enzyme

assay. This finding ruled out the possibility that the activator was cysteine, cystine, GSH or GSSG, since these compounds were required at concentrations near 0.10 mM to produce a similar activation of the enzyme.

Activator purified from cells grown on [^{35}S]-sulphate contained no detectable radioactivity. From the known specific radioactivity of the ^{35}S in the medium (6.85 $\mu\text{Ci}/\text{mg-atom}$ of S) it was calculated that sulphur was present at less than 3 μM in purified activator extracts, i.e. if the activator was a sulphur compound it would need to have been active at less than 0.3 μM in the enzyme assay.

Purified activator preparations were also assayed for total phosphate and aromatic amines, but no reactivity was observed. Both these methods could detect 5 nmol of compound, so that if the activator contained phosphate or an aromatic amino group it must have been active at a concentration below 1 μM in the enzyme assay. Purified activator solutions exhibited no characteristic u.v.-absorption spectrum.

Purified activator preparations were treated with dansyl chloride as described in the Materials and Methods section. Dansylated extracts could be assayed for activator by the addition of substrates and enzyme directly to the dansylation reaction tube, since the concentration of reagents was sufficiently low not to affect the enzyme assay. Generally 50–70% of the activity was recovered after dansylation. Although the activator was relatively stable to dansylation, it was very unstable to subsequent fractionation. No activity was recovered when dansylated activator was fractionated by gel filtration, solvent extraction, high-voltage electrophoresis or t.l.c. This marked loss of stability on dansylation, together with the evidence for the presence of an amino group in the molecule, makes it reasonable to assume that the activator did form a dansyl derivative. T.l.c. of dansylated extracts was performed on polyamide layers in the solvent systems described by Woods & Wang (1967). Purified activator extracts contained only one major dansyl derivative, which was present in all active preparations. The concentration of this derivative in dansylated activator extracts was determined by a comparison of fluorescence intensity with known amounts of standard dansyl amino acids. Since the method could detect 0.01 nmol of dansyl amino acid, the compound represented by the major dansyl derivative was present in activator extracts at a concentration of 0.01 μM .

The behaviour of the activator on ion-exchange resins, phosphocellulose, electrophoresis and paper chromatography was similar to that of pteridines in these systems (Blakley, 1969). The fluorescence spectrum of purified activator preparations obtained at neutral pH in an Aminco-Bowman spectrophotofluorimeter was essentially identical with that of authentic 2-amino-4-hydroxy-6-methylpteridine. It

was found, however, from the fluorescence assay (excitation 365 nm, emission 445 nm) of fractions from the phosphocellulose column described in Fig. 4, that the elution profile for pteridines was not exactly correlated with the activator activity. The total pteridine concentration in purified activator samples was determined by fluorescence and calculation showed that if the activator was a pteridine then it must be active at 0.60 μM in the enzyme assay.

The pteridines listed in the Materials and Methods section were tested for activator activity in the concentration range 0.25 μM –1.0 mM. Only one of the compounds produced a significant effect; 6-hydroxymethylpterin, at 0.2 mM, activated the enzyme 4-fold. None of the pteridines at 0.1 mM was effective as an inhibitor of the activation with endogenous activator.

Interaction of activator and aminolaevulinate synthetase

Activator was determined as a routine by the addition of extracts to the assay mixture for crude low-activity enzyme (Fig. 1). To clarify the mechanism of this activation the effect of purified activator (from phosphocellulose) on successively purified preparations of low-activity enzyme was studied. A four-step purification of low-activity enzyme and the preparation of the pellet factor was described in the preceding paper (Neuberger *et al.*, 1973).

Table 1 shows the effect of optimum concentrations of activator and activator plus pellet factor on the activity of the enzyme after each purification step. The mechanism of the activation with activator was different from that previously established with sulphur compounds (Neuberger *et al.*, 1973) (but see 'Note added in proof'). With sulphur compounds, activation was absolutely dependent on the pellet factor and, after purification of the enzyme on Sephadex G-100, sulphur compounds were ineffective even in the presence of the pellet factor. The activation with activator, however, though enhanced by the addition of the pellet factor, was not absolutely dependent on this factor. Also, 3–4-fold activation was obtained with activator even after purification of the enzyme on Sephadex G-100. Further, activation of the Sephadex G-100 eluate with activator was essentially unaffected by the exclusion of air; all activations with sulphur compounds, however, were absolutely dependent on a supply of air. These results indicated that, in contrast with sulphur compounds, the endogenous activator produced activation by direct interaction with the enzyme. In common with sulphur compounds the activator was shown to affect the low-activity form of the enzyme only. Thus activator enhanced the activity of crude low-activity enzyme

Table 1. *Effect of activator 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, purified activator (10 μ l) and pellet factor (10 μ l) (prepared as described in the Materials and Methods section) as indicated. Incubation was at 37°C for 30–90 min and the aminolaevulinate synthetase activity was determined. N.d., not determined.

Enzyme preparation	Additions ...	Aminolaevulinate synthetase activity (units/mg of protein)			
		None	Activator	Pellet factor	Activator and pellet factor
1. Crude extract		35	208	N.d.	N.d.
2. High-speed supernatant (pigment-free)		62	242	70	265
3. DEAE-cellulose eluate		82	302	88	342
4. Sephadex G-100 eluate		275	922	262	1220

Fig. 6. *Effect of activator on the activity of purified low-activity aminolaevulinate synthetase in the absence of substrates*

Tubes containing enzyme (about 100 μ g of protein; Step 4 of purification; see the Materials and Methods section) and purified activator (10 μ l) were incubated at 22°C. At intervals substrate mixture was added and aminolaevulinate synthetase activity was determined by incubation at 37°C for 60 min (●). A set of control tubes was included with enzyme incubated alone at 22°C, and activator was added along with the substrate mixture (▲).

Table 2. *Effect of individual aminolaevulinate synthetase substrates on the inactivation of purified enzyme by activator*

Tubes, containing enzyme (about 100 μ g of protein; Step 4 of purification procedure; see the Materials and Methods section), purified activator (10 μ l), the substrates listed and water to make a total volume of 0.25 ml, were incubated at 22°C. Substrates were present at four times their concentration in the normal aminolaevulinate synthetase assay. After incubation at 22°C for 90 min the other substrates were added where required, water was added to make a total volume of 1.0 ml, and the aminolaevulinate synthetase activity was determined by incubation at 37°C for 60 min.

Addition to enzyme for preincubation	Aminolaevulinate synthetase activity (units/mg of protein)
None	275
Activator	147
Activator, glycine	175
Activator, CoA	192
Activator, pyridoxal phosphate	764
Activator, succinate	803
Activator, MgCl ₂ , ATP	160
Activator, glycine, CoA, pyridoxal phosphate, succinate, MgCl ₂ , ATP	845

and also the activity of the low-activity form observed in extracts of semi-anaerobically grown cells immediately after cell disruption. However, the activator produced no significant effect on the activity of crude

high-activity enzyme (prepared as described in the Materials and Methods section).

A further indication that the purified activator interacted directly with aminolaevulinate synthetase

was obtained when purified low-activity enzyme and activator were incubated at 22°C in the absence of enzyme substrates. The activator produced a time-dependent decay of aminolaevulinate synthetase activity (Fig. 6). This decay was not due to an inherent instability of the enzyme, since incubation in the absence of activator produced no significant loss in activity. The rate of decay in the presence of activator was also a temperature-dependent process. Thus, when the experiment described in Fig. 6 was done at 4°C instead of at 22°C, the specific activity had fallen from 885 to 440 units/mg of protein after 2 h. When the experiment was done at 37°C, total loss of activity was observed after only 75 min. The rate of decay of enzyme activity in the absence of activator was also much faster at higher temperatures, so that the effect produced by activator was most marked when the experiment was done at 22°C. Enzyme that had been inactivated by activator in the absence of substrates could not be re-activated by the addition of more activator in the presence of substrates.

When enzyme and activator were incubated together at 22°C in the presence of each of the aminolaevulinate synthetase substrates individually, the rapid decay process was almost completely prevented by the presence of either pyridoxal phosphate or sodium succinate (Table 2). None of the other substrates alone gave significant protection.

Discussion

Alterations in aminolaevulinate synthetase activity in vitro

It has been shown by a number of workers that the aminolaevulinate synthetase of *R. spheroides* can be found in extracts in two forms of differing activities. The enzyme from cells grown under semi-anaerobic conditions was present in a low-activity form immediately after cell disruption and was converted into a high-activity form on storage of the extracts in the presence of air or light (Marriott, 1968; Tuboi *et al.*, 1969). Marriott *et al.* (1969) showed that activation involved endogenous compounds of low molecular weight and they later concluded (Marriott *et al.*, 1970) that activation occurred as a direct consequence of some endogenous factor being oxidized via the electron-transport chain. The aminolaevulinate synthetase in crude extracts of *R. spheroides* was activated 3–4-fold by the addition of potassium ferricyanide (0.5 mM) and inhibited by 75% by 1.0 mM-dithiothreitol (Warnick & Burnham, 1971). Since these effects were not observed with the purified enzyme, it was suggested that *R. spheroides* might contain a redox-sensitive regulator of aminolaevulinate synthetase activity.

Tuboi & Hayasaka (1972a) partially purified low- and high-activity forms of aminolaevulinate synthetase from *R. spheroides* and were able to convert the

former into the latter by adding any of a number of disulphide compounds, cystine being the most effective. This effect of sulphur compounds seems to be mediated by an endogenous activating enzyme (Tuboi & Hayasaka, 1972b). In the preceding paper we reported the activation of aminolaevulinate synthetase in crude extracts by both disulphide and thiol compounds. Activation was dependent on a particulate fraction isolated from *R. spheroides* and a supply of air. When the enzyme was purified activation could no longer be demonstrated with the sulphur compounds tested, even in the presence of air and of the particulate fraction; we concluded that other factors must be involved. In the present paper we have described the properties of an activator of aminolaevulinate synthetase which was isolated from extracts of semi-anaerobically grown *R. spheroides*. This activator, in contrast with the sulphur compounds tested, does not appear to require for activation of the enzyme other factors or a supply of air. It appears that this activator interacts directly with aminolaevulinate synthetase in purified preparations.

Nature of the endogenous activator

A physiological role for the activator is indicated by the extremely low concentration required to produce marked changes in the activity of aminolaevulinate synthetase. Although the behaviour of the activator in a variety of chromatographic systems and on electrophoresis has been determined, its chemical nature is still unknown (but see 'Note added in proof'). It is an amino compound of low molecular weight, the activity of which is stable to acid treatment but destroyed by alkali. The activity also disappears on reduction with NaBH₄ or Na₂S₂O₄ or on oxidation with performic acid; oxidation with potassium ferricyanide or I₂–KI does not affect the activity. Many of the properties of the activator are similar to those of pteridines and the most highly purified preparations of activator contain pteridines. However, on chromatography on phosphocellulose, the elution pattern of activator is different from that of the pteridines also present in the extract.

Control of aminolaevulinate synthetase in R. spheroides

There is evidence (cf. Lascelles, 1968) that both the activity and the synthesis of aminolaevulinate synthetase in *R. spheroides* is controlled by the intracellular content of free haem. However, an increasing number of reports indicate that the activity of the enzyme is also controlled by the concentrations or oxidation states of non-haem factors in the cell.

From the present work it can be suggested that aminolaevulinate synthetase in *R. spheroides* is directly controlled by the intracellular concentration of the oxidized form of an endogenous activator of the

enzyme. This activator appears to be inactive in its reduced form. Further, the redox state of the activator seems to be controlled by an enzyme-dependent thiol-disulphide couple linked to the electron-transport chain; however, the situation may be more complex and the possible specific effect of oxygen on the activator is considered below.

Evidence has been presented (cf. Fig. 6 and Table 2) that the endogenous activator studied in the present paper interacts directly with the enzyme. The conversion of aminolaevulinate synthetase into the low-activity form on harvesting (Neuberger *et al.*, 1973) is probably due to a fall in the intracellular concentration of the oxidized form of the activator. During harvesting it is known that there is a loss of intracellular oxidizing potential (Cohen-Bazire *et al.*, 1957; Wimpenny & Firth, 1972). The spontaneous activation (Marriott, 1968) would then be due to a time-dependent reoxidation of the accumulated reduced activator, as would the activating effects of either thiol or disulphide compounds in the presence of oxygen (Neuberger *et al.*, 1973). The activation of the enzyme in crude extracts by chemical oxidants (Warnick & Burnham, 1971) can also be explained by oxidation of reduced endogenous activator.

The inactivation of aminolaevulinate synthetase produced by oxygenation of *R. sphaeroides* growing semi-anaerobically in the light and the inability of aminolaevulinate synthetase to become activated in extracts of oxygenated cells is more difficult to explain. Inactivation may result from the intracellular depletion of thiol and disulphide compounds that occurs during oxygenation (Neuberger *et al.*, 1973); in addition, oxygenation may lead to direct inactivation of the activator. Further consideration of the mechanism of inactivation must await the identification of the endogenous activator.

Note added in proof (received 10 September 1973)

More recent experiments have strongly suggested that the activator is a trisulphide of cystine (thiocystine) or a trisulphide of GSSG or a closely related

compound. These results have been communicated in a recent paper (Davies *et al.*, 1973). The term 'sulphur compounds' used in the present and the preceding paper thus refers to thiols and disulphides tested but does not include trisulphides.

Some of this work forms part of a Ph.D. Thesis submitted to the University of London in January 1973 by J. D. S. We are grateful to Mr. S. Ladeji for his excellent technical assistance.

References

- Blakley, R. L. (1969) *The Biochemistry of Folic Acid and Related Pteridines: Frontiers of Biology*, vol. 13, pp. 58–98, North-Holland, Amsterdam and London.
- Cohen-Bazire, G., Sistrom, W. R. & Stanier, R. Y. (1957) *J. Cell. Comp. Physiol.* **49**, 25–68.
- Davies, R. C., Gorchain, A., Neuberger, A., Sandy, J. D. & Tait, G. H. (1973) *Nature (London)* **245**, in the press.
- Lascelles, J. (1968) *Biochem. Soc. Symp.* **28**, 49–59.
- Marriott, J. (1968) *Biochem. Soc. Symp.* **28**, 61–74.
- Marriott, J., Neuberger, A. & Tait, G. H. (1969) *Biochem. J.* **111**, 385–394.
- Marriott, J., Neuberger, A. & Tait, G. H. (1970) *Biochem. J.* **117**, 609–613.
- Neuberger, A., Sandy, J. D. & Tait, G. H. (1973) *Biochem. J.* **136**, 477–490.
- Peterson, E. A. & Sober, H. A. (1956) *J. Amer. Chem. Soc.* **78**, 751–755.
- Rembold, H. & Buschmann, L. (1962) *Hoppe-Seyler's Z. Physiol. Chem.* **330**, 132–139.
- Rosen, H. (1957) *Arch. Biochem. Biophys.* **67**, 10–15.
- Tuboi, S., Haeng, J. K. & Kikuchi, G. (1969) *Arch. Biochem. Biophys.* **130**, 92–100.
- Tuboi, S. & Hayasaka, S. (1972a) *Arch. Biochem. Biophys.* **150**, 690–697.
- Tuboi, S. & Hayasaka, S. (1972b) *J. Biochem. (Tokyo)* **72**, 219–222.
- Warnick, G. R. & Burnham, B. F. (1971) *J. Biol. Chem.* **246**, 6880–6885.
- Wimpenny, J. W. T. & Firth, A. (1972) *J. Bacteriol.* **111**, 24–32.
- Woods, K. R. & Wang, K. T. (1967) *Biochim. Biophys. Acta* **133**, 369–370.