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Control of Porphyrin Biosynthesis through a Negative-Feedback Mechanism

STUDIES WITH PREPARATIONS OF δ-AMINOLAEVULATE SYNTHETASE AND δ-AMINOLAEVULATE DEHYDRATASE FROM *RHODOPSEUDOMONAS SPHEROIDES*

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The photosynthetic bacterium *Rhodopseudomonas spheroides*, like many other micro-organisms, accumulates porphyrins in the medium when cultured under iron-deficient conditions (Lascelles, 1961). The addition of small amounts of iron (2 μm-moles/ml.) to suspensions of iron-deficient *R. spheroides* causes a large decrease (up to 100 μm-moles/ml.) in the quantity of porphyrin produced by the organism. Iron cannot therefore be acting solely by diverting porphyrins (or precursors) to iron porphyrins since the quantity of porphyrin that fails to accumulate in the presence of iron is about 100 times more than the amount of iron required to suppress the excretion (Lascelles, 1956).

Iron may act catalytically on porphyrin excretion by promoting the synthesis of a haem compound that in turn controls the activity of an enzyme concerned in porphyrin synthesis through a negative-feedback mechanism. In other negative-feedback systems the final product in a metabolic sequence inhibits the first enzymic step that leads directly to that end product (Umbarger, 1961). In porphyrin synthesis the enzyme that fills this position is δ-aminolaevulate synthetase, which catalyses the formation of δ-aminolaevulate from glycine and succinyl-CoA (Kikuchi, Kumar, Talmage & Shemin, 1958; Gibson, 1958).

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Previous work has shown that haemin represses the formation of δ-aminolaevulate synthetase in *R. spheroides* (Lascelles, 1960) and that haemin inhibits the action of the enzyme in crude cell extracts (Gibson, Matthew, Neuberger & Tait, 1961). In the present investigation the possibility that haemin exerts a negative-feedback type of control on δ-aminolaevulate synthetase has been examined by studying its action on partially purified preparations of the enzyme from *R. spheroides*. Evidence consistent with such a controlling function has also been shown with suspensions of the organism that produce porphyrins. Preliminary accounts of this work have appeared (Burnham, 1962*a*, *b*). The purification of succinyl-coenzyme A thiokinase (used in the assay for δ-aminolaevulate synthetase) and of δ-aminolaevulate dehydratase from *R. spheroides* is also described.

EXPERIMENTAL

Chemicals

CoA (75%), ATP, pyridoxal phosphate, horse-heart cytochrome *c* and twice-recrystallized bovine haemoglobin were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Succinyl-CoA was prepared by the method of Simon & Shemin (1953) and was used immediately. The values quoted for succinyl-CoA are based on the amount of CoA

(assumed to be 75% pure) used. Alternatively, succinyl-CoA was generated in the reaction mixture from succinate, ATP and CoA by succinyl-coenzyme A thiokinase.

Phosphate buffers were made from KH_2PO_4 and K_2HPO_4. Tris buffers were prepared according to Gomori (1955). Other buffers were prepared by adjusting the pH of 0·2M solutions with N-KOH or N-HCl.

Protohaemin IX was obtained from Schuchardt G.m.b.H., Munich, Germany. A portion of this haemin was recrystallized three times before being used in inhibition studies. The results obtained were indistinguishable from those obtained when the haemin was used without this precautionary purification.

Coproporphyrin III was obtained from culture supernatants of iron-deficient *R. spheroides* (Lascelles, 1956).

Protoporphyrin and deuteroporphyrin were prepared by the method of Fischer & Putzer (1926) and Fischer, Treibs & Zeile (1931). Haematoporphyrin was purchased from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.

All these porphyrins were converted into their methyl esters, and were chromatographed on $CaCO_3$ with benzene and chloroform (Falk, 1961). Melting points (uncorrected) of the crystalline fractions after chromatography were (with melting points given in the literature in parentheses): haematoporphyrin dimethyl ester, 209° (212°); deuteroporphyrin dimethyl ester, 220° (224°); protoporphyrin dimethyl ester, 220° (225–230°); coproporphyrin tetramethyl ester, 122° (130–158°) and 160° (150–179°). Before use, the esters were hydrolysed in 6N-HCl and subsequently twice evaporated to dryness *in vacuo* over solid KOH.

Metals were incorporated into the porphyrins as the corresponding acetates (Schwartz, Berg, Bossenmaier & Dinsmore, 1960). Within 1 hr. before use, mM stock solutions were prepared as follows. The crystalline iron porphyrin was dissolved in 0·5 ml. of N-KOH and diluted to 5·5 ml. with freshly boiled water and 0·5 ml. of M-tris buffer, pH 7·8. The solution was gently mixed while 0·4 ml. of N-HCl was slowly added, and the volume was made up to 10 ml. with freshly boiled water. The solutions to be used in the experiments were then prepared from this stock solution by using freshly boiled water.

Crystalline bacteriochlorophyll and bacteriochlorophyllide were prepared from *R. spheroides* by a method based on that of Jacobs, Vatter & Holt (1954). These were dissolved in 50% (v/v) methanol. Magnesium protoporphyrin was prepared by the method of Granick (1948). Crystalline horse-liver catalase was prepared by the method of Bonnichsen (1947). Crystalline horse myoglobin was generously supplied by Å. Åkeson of the Nobel Medical Institute, Stockholm, Sweden. Native globin was prepared from bovine haemoglobin by the method of Rossi-Fanelli, Antonini & Caputo (1958).

Organism

The strain of *R. spheroides* used (N.C.I.B. 8253, from the National Collection of Industrial Bacteria) was that described by Lascelles (1956).

Growth and harvesting

The culture medium ('medium MS') was that described by Lascelles (1959). The organisms were grown photosynthetically, harvested by centrifuging at the end of the logarithmic phase of growth (14–20 hr.) and were washed once with 0·1M-tris buffer, pH 7·8. They were finally suspended at the required concentration in 0·1M-tris buffer, pH 7·8, and either stored at −20° or used directly. In one phase of the investigation cells were grown by Mr R. Elsworth of the Microbiological Research Establishment, Porton, Wilts. These cells were grown aerobically under low aeration on medium MS supplemented with acetate.

Preparation of cell-free extracts

Suspensions containing 10–50 mg. dry wt. of organisms/ml. were disrupted by ultrasonic vibration for 5 min. at 25 kcyc./sec. with a Mullard ultrasonic generator type E 7590B (Mullard Ltd., London, W.C. 1), cooled by a rapidly flowing current of cold water. Whole cells and cell debris were removed by centrifuging for 10 min. at 34 000*g* at 2° in a Servall centrifuge.

Determinations

Dry weight of organisms. The extinction of suspensions was measured at 680 mμ and the dry weight calculated from a calibration curve; the absorption due to photosynthetic pigments is minimal at this wavelength (Cohen-Bazire, Sistrom & Stanier, 1957).

Protein. The concentration of protein in extracts was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Crystalline bovine plasma albumin (Armour Laboratories, Sussex) was used as the standard.

Bacteriochlorophyll and porphyrins. The bacteriochlorophyll concentration in whole cells was determined by the method of Cohen-Bazire *et al.* (1957); porphyrins accumulated in the medium were determined as described by Lascelles (1956).

Assay of enzymic activities

δ-Aminolaevulate synthetase. δ-Aminolaevulate-synthetase activity was measured in a standard assay mixture containing: glycine, 100 μmoles; sodium succinate, 100 μmoles; CoA, 0·58 μmole; pyridoxal phosphate, 0·25 μmole; ATP, 7·5 μmoles; $MgCl_2$, 10 μmoles; tris buffer, pH 7·8, 50 μmoles; EDTA, 1·0 μmole; β-mercaptoethanol, 1·0 μmole; succinyl-coenzyme A thiokinase, 0·1 ml. (specific activity 520 μmoles of succinyl-CoA/hr./mg.); inhibitors as indicated; partially purified δ-aminolaevulate synthetase, 0·1 ml. (about 4 mg. of protein); water to give a final volume of 1·0 ml. Incubation was at 37° for 30 min. in 13 mm. tubes. The reaction was terminated by adding 1 ml. of 10% (w/v) trichloroacetic acid and the protein was removed by centrifuging. The δ-aminolaevulate in a sample of the supernatant was determined by the method of Mauzerall & Granick (1956). In experiments with the more highly purified preparations of enzyme there was no advantage in adding either EDTA or β-mercaptoethanol, and they were usually omitted.

δ-Aminolaevulate dehydratase. This enzyme was assayed by a slightly modified version of the method of Gibson, Neuberger & Scott (1955). The system contained: potassium phosphate buffer, pH 7·5, 125 μmoles; $MgCl_2$, 5 μmoles; L-cysteine, 25 μmoles; δ-aminolaevulate, 5 μmoles; inhibitors, as indicated; enzyme, as indicated; water to give a final volume of 2·5 ml. The enzyme was preincubated in the reaction mixture for 10 min. before the addition of δ-aminolaevulate. Incubation was at 37° for

30 min. The reaction was stopped by the addition of 0·1 ml. of 25% (w/v) $CuSO_4,5H_2O$, the precipitated material was centrifuged and porphobilinogen was determined in the supernatant by the method of Mauzerall & Granick (1956).

Succinyl-coenzyme A thiokinase. This enzyme was assayed by a method similar to that of Kaufman & Alivisatos (1955). The system contained: sodium succinate, 100 μmoles; CoA, 0·58 μmole; ATP, 7·5 μmoles; L-cysteine or β-mercaptoethanol, 10 μmoles; hydroxylamine (added as hydroxylamine hydrochloride–KOH mixture, pH 7·4) 800 μmoles; enzyme; water to give a final volume of 1·0 ml. Incubation was at 37° for 30 min. in 13 mm. tubes. Succinohydroxamate was determined by the method of Lipmann & Tuttle (1945).

Purification of succinyl-coenzyme A thiokinase

A suspension (200 ml.) of *R. spheroides* containing 50 mg. dry wt. of organisms/ml. was used as the starting material. All operations were carried out at 2° unless stated otherwise.

Step 1. *Preparation of extract.* Samples (20 ml.) of cells were disrupted by ultrasonic treatment and centrifuged for 20 min. at 34 000*g*.

Step 2. *Preliminary ammonium sulphate fractionation.* The crude supernatant from step 1 was collected and solid $(NH_4)_2SO_4$ was added to give 25% saturation. After equilibration for 30 min., the precipitate was removed by centrifuging and discarded. Solid $(NH_4)_2SO_4$ was slowly added to the supernatant to give 65% saturation. After equilibration for 30 min., the precipitate was removed by centrifuging and the supernatant discarded.

Step 3. *Protamine sulphate fractionation.* The precipitate from step 2 was dissolved in 50 ml. of 0·2 M-potassium phosphate buffer, pH 6·8, and dialysed against two changes of water for 24 hr. at 2°. After dialysis, the solution was centrifuged to remove insoluble matter, and the pH of the supernatant adjusted to 6·0 with M-acetate buffer. A solution of protamine sulphate (20 mg./ml.) was added slowly with mixing until a total of 40 ml. had been added. The solution was equilibrated at 2° for 1 hr., with occasional stirring, before the copious precipitate was removed by centrifuging.

Step 4. *Removal of excess of protamine sulphate.* Solid $(NH_4)_2SO_4$ was added to the supernatant to give 60% saturation and the mixture equilibrated for 30 min. before centrifuging. The supernatant containing excess of protamine sulphate was discarded, and the precipitate was dissolved in 10 ml. of 0·1 M-tris buffer, pH 7·8. This solution was chilled to −3°, and was kept at this temperature for 10 min., then centrifuged for 10 min. at −3° at 17 000*g*. The supernatant was immediately decanted.

Step 5. *Final ammonium sulphate fractionation.* The supernatant was adjusted to 30% saturation with solid $(NH_4)_2SO_4$. The precipitate was collected by centrifuging and dissolved in 5 ml. of 0·1 M-tris buffer, pH 7·8. This preparation could be kept at 1° for at least 15 days with essentially no loss in activity.

Further purification by chromatography on diethylaminoethylcellulose. Further purification of succinyl-coenzyme A thiokinase could be achieved by chromatography on diethylaminoethylcellulose (DEAE-cellulose). The enzyme extract was adsorbed on a column (1·8 cm. × 18 cm.) at pH 7·9 in mM-tris buffer. Elution was carried out by increasing the ionic strength with KCl, either stepwise or with a linear gradient. The enzyme was eluted with approx. 0·3 M-KCl. Difficulty was occasionally encountered in adsorbing the enzyme on the DEAE-cellulose. However, successful runs yielded an enzyme with a specific activity of up to 520 μmoles of succinyl-CoA/hr./mg. of protein. After DEAE-cellulose chromatography the enzyme could be stored at −20° with little loss in activity over several months.

The purification procedure is summarized in Table 1.

Preparation of δ-aminolaevulate synthetase

Suspensions of *R. spheroides* containing 50 mg. dry wt. of cells/ml. in 0·04 M-tris buffer, pH 7·8, were divided into 20 ml. samples, frozen and stored at −20°. Such preparations provided a relatively stable and uniform starting material for the purification experiments. All operations were carried out at 2° unless stated otherwise.

Step 1. *Preparation of crude enzyme extract.* After the ultrasonic treatment, 15 ml. of the broken-cell suspension was centrifuged at 2° for 30 min. at 34 000*g*. The precipitated cell debris was discarded and 2 ml. of 5 mM-*o*-phenanthroline, 0·1 ml. of 0·1 M-β-mercaptoethanol and 1 ml. of 0·1 M-$MgCl_2$ were added to 10 ml. of the supernatant.

Step 2. *Acetone precipitation.* The extract from step 1 was slowly added to 70 ml. of acetone that had been cooled in solid CO_2 to −40°. The mixture was left for 10 min. before centrifuging. The precipitate was resuspended in 20 ml. of acetone at −40° and was centrifuged again, this time the precipitate being collected in a single centrifuge tube.

Table 1. *Purification of succinyl-coenzyme A thiokinase*

Experimental details are given in the text.

Fraction	Volume (ml.)	Concn. of protein (mg./ml.)	Specific activity (μmoles of succinohydroxamate/hr./mg. of protein)
Crude ultrasonically-treated extract	200	26	2·9
Redissolved precipitate from treatment with 65% saturated $(NH_4)_2SO_4$, after dialysis	92	15	4·2
Supernatant from treatment with protamine sulphate	130	3	16
Redissolved precipitate from treatment with 60% saturated $(NH_4)_2SO_4$	10	24	17·9
Redissolved precipitate from treatment with 30% saturated $(NH_4)_2SO_4$	5	10·2	28·9
Eluate from DEAE-cellulose column	—	0·49	520

Table 2. *Purification of δ-aminolaevulate synthetase*

Experimental details are given in the text.

Expt. no.	Preparation	Volume (ml.)	Concn. of protein (mg./ml.)	Specific activity (μm-moles of δ-aminolaevulate/ hr./mg. of protein)
1	Crude ultrasonically-treated extract	15	24	205
	Redissolved precipitate from treatment with acetone	10	10	460
	Redissolved precipitate from treatment with 50% saturated $(NH_4)_2SO_4$	8	1·9	2000
2	Crude ultrasonically-treated extract	20	17·8	170
	Redissolved precipitate from treatment with acetone	10	6·4	680
	Redissolved precipitate from treatment with 50% saturated $(NH_4)_2SO_4$	5	4·4	1625

Step 3. *Ammonium sulphate precipitation.* The precipitate from step 2 was resuspended in 10 ml. of 0·1 M-tris buffer, pH 7·8, containing β-mercaptoethanol (2 mM) and EDTA (2 mM). After being mixed for 15 min. the suspension was centrifuged and the precipitate discarded. To 8 ml. of this supernatant, 0·2 ml. of 0·1 M-β-mercaptoethanol, 0·8 ml. of 5 mM-*o*-phenanthroline, 0·4 ml. of 0·1 M-$MgCl_2$ and 0·2 ml. of 0·1 M-EDTA were added. An equal volume of saturated $(NH_4)_2SO_4$ (at room temperature) was added slowly to this mixture, which was held in an ice bath to keep the temperature below 5°. After equilibration for 20 min. the precipitate was collected by centrifuging, and the supernatant discarded. The precipitate was redissolved in 8 ml. of 0·1 M-tris buffer, pH 7·8, and was then dialysed against 0·01 M-tris for several hours.

The partially purified δ-aminolaevulate synthetase had a specific activity of about 1800 μm-moles of δ-aminolaevulate/hr./mg. of protein. The yield and activity did not generally vary by more than a few per cent. The results obtained in two such preparations are summarized in Table 2. The stability of the different preparations, however, did vary somewhat, though preparations retained about 80% of their activity for about 5 days when stored at 1°.

Repeated attempts to effect further purification of the enzyme with DEAE-cellulose were disappointing: whereas the behaviour of the enzyme in the early stages of purification seemed predictable, it became increasingly erratic with further purification. Likewise, when the described purification procedure was carried out with 5 or 10 times the amounts of materials, it proved to be less reliable.

Purification of δ-aminolaevulate dehydratase

Cells used for the purification of this enzyme were grown at the Microbiological Research Establishment, Porton, Wilts. All fractionations were carried out at 2° unless stated otherwise.

Step 1. *Preparation of crude enzyme extract.* A thick cell paste containing 77 g. wet wt. of cells was mixed with enough 0·1 M-tris buffer, pH 7·8, to give a mixture just thin enough to be poured. This material was passed once through a French Pressure Cell (American Instrument Co., Silver Spring, Md., U.S.A.). The crushed preparation was treated ultrasonically for 3 min. in 20 ml. portions to denature the nucleic acid released from the disrupted cells. After the ultrasonic treatment there was a large decrease in viscosity. The ultrasonically treated extract was diluted to 300 ml. with 0·1 M-tris buffer, pH 7·8, containing EDTA (mM) and β-mercaptoethanol (mM). This extract was centrifuged for 90 min. in a Spinco model L ultracentrifuge at 78 400*g*.

Step 2. *Acetone precipitation.* The supernatants from step 1 were combined and 10 ml. of 0·1 M-$MgCl_2$ plus 1 ml. of 0·01 M-β-mercaptoethanol were added. After being mixed, the extract was slowly added to 1 l. of acetone at −20°. The precipitate was removed by centrifuging at −10°, and was washed once with an additional 200 ml. of cold acetone. The washed acetone precipitate was redissolved in 60 ml. of 0·1 M-tris buffer, pH 7·8, containing EDTA (mM) and β-mercaptoethanol (mM). The mixture was dialysed for 20 hr. against changes of 0·01 M-tris buffer, pH 7·8, containing EDTA (mM) and β-mercaptoethanol (mM). After dialysis, the insoluble residue was removed by centrifuging.

Step 3. *Ammonium sulphate fractionation.* Solid $(NH_4)_2SO_4$ was added to the supernatant to give increments of 10% saturation. After each addition, the solution was equilibrated for 30 min. before centrifuging. Each precipitate collected during this treatment was redissolved in 10 ml. of 0·1 M-tris buffer, pH 7·8, and was dialysed overnight against the same buffer. The fraction that was precipitated at 40% saturation contained essentially all the δ-aminolaevulate-dehydratase activity.

Step 4. *Chromatography on diethylaminoethylcellulose.* The active fraction from step 3 was further dialysed against 2 mM-tris buffer, pH 7·8, containing EDTA (mM) and β-mercaptoethanol (mM). After dialysis, this partially purified enzyme extract was placed on a column (1·2 cm. × 20 cm.) of DEAE-cellulose equilibrated with the same buffer. Protein was eluted from the column by increasing the ionic strength of the eluting buffer with M-KCl in a stepwise manner.The δ-aminolaevulate dehydratase was eluted in the presence of about 0·18 M-KCl.

The results obtained with this procedure are summarized in Table 3.

RESULTS

Properties of δ-aminolaevulate synthetase

Under standard assay conditions, the amount of δ-aminolaevulate formed was linear with protein concentration up to 0·8 mg./ml. and with time for at least 50 min.

Effect of pH *on enzyme activity.* Although there was no apparent sharp optimum pH, maximal activity was observed between pH 7·8 and pH 8·0

(Fig. 1). The activity in tris, glycylglycine and triethanolamine buffers was essentially the same, but in phosphate buffer it was between 50 and 60% of that in the first group.

Michaelis constant. A Michaelis constant, K_m, of 280 μM for glycine was calculated from a Lineweaver & Burk (1934) plot (Fig. 2).

It was not possible to obtain a satisfactory plot of enzyme activity as a function of pyridoxal phosphate concentration, owing to difficulties in resolving this cofactor and the enzyme. However, several attempts indicated that K_m for this cofactor was about 4 μM.

With chemically prepared succinyl-CoA as the substrate for δ-aminolaevulate synthetase, in the place of succinate, ATP and succinyl-CoA thiokinase, the formation of δ-aminolaevulate as a function of enzyme concentration remained linear

Table 3. *Purification of δ-aminolaevulate dehydratase*

Experimental details are given in the text.

Fraction	Protein concn. (mg./ml.)	Specific activity (μm-moles of porphobilinogen/hr./mg. of protein)
Supernatant obtained from centrifuging at 78 400*g*	16·6	89·5
Redissolved precipitate obtained from treatment with 40% saturated $(NH_4)_2SO_4$	9·7	520
Eluate from DEAE-cellulose column	0·43	6000

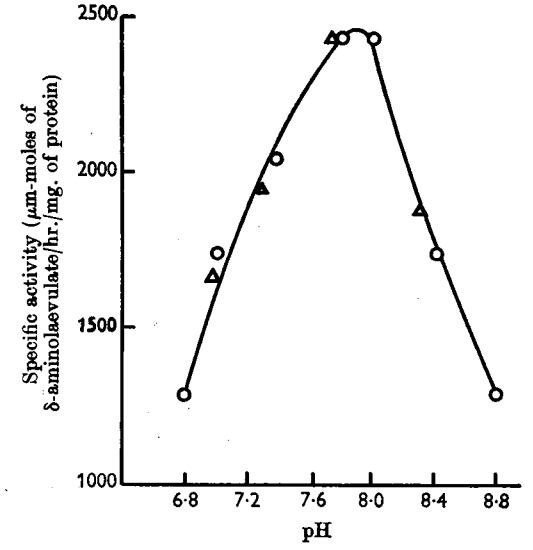

Fig. 1. δ-Aminolaevulate-synthetase activity as a function of pH. The incubation mixture contained: glycine (100 μmoles), sodium succinate (100 μmoles), CoA (0·58 μmole), pyridoxal phosphate (0·25 μmole), ATP (7·5 μmoles), $MgCl_2$ (10 μmoles), buffer (50 μmoles), succinyl-coenzyme A thiokinase (0·1 ml.), enzyme (0·1 ml.) and water to give a final volume of 1·0 ml. The buffers used were: △, tris; ○, triethanolamine. Incubations were at 37° for 30 min. The reaction was stopped with trichloroacetic acid, and δ-aminolaevulate was determined.

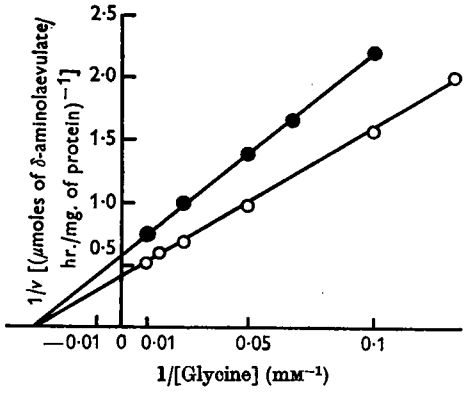

Fig. 2. Michaelis constant for δ-aminolaevulate synthetase. The glycine concentration was varied while the other reaction components were held constant as described in the experimental sections. ○, Reaction without inhibitor; ●, reaction in the presence of μM-haemin.

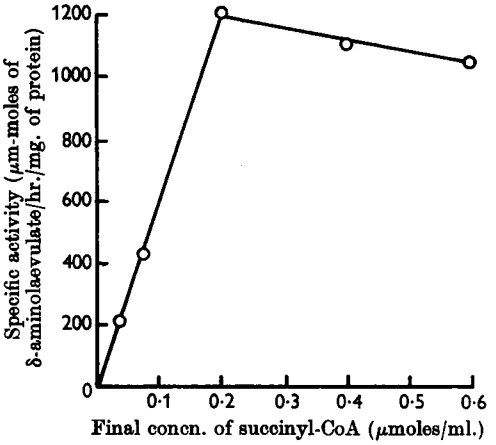

Fig. 3. Synthesis of δ-aminolaevulate as a function of succinyl-CoA concentration. The incubation mixture contained: glycine (100 μmoles), CoA (0·58 μmole), pyridoxal phosphate (0·25 μmole), tris buffer, pH 7·8 (50 μmoles), succinyl-CoA (as shown), enzyme (0·1 ml.) and water to give a final volume of 1·0 ml. Incubations were at 37° for 30 min. The reaction was stopped with trichloroacetic acid, and δ-aminolaevulate was determined.

Table 4. *Effect of metal ions and general enzyme inhibitors on δ-aminolaevulate synthetase*

The incubation mixtures contained: glycine (100 μmoles), sodium succinate (100 μmoles), CoA (0·58 μmole), pyridoxal phosphate (0·25 μmole), ATP (7·5 μmoles), $MgCl_2$ (10 μmoles), tris buffer, pH 7·8 (50 μmoles), succinyl-coenzyme A thiokinase (0·1 ml.), enzyme (0·1 ml.) and other additions (final concentrations given in parentheses) to 1·0 ml. were incubated at 37° for 30 min. The reaction was stopped with trichloroacetic acid, and δ-aminolaevulate was determined.

Additions	Specific activity (μm-moles of δ-aminolaevulate/hr./mg. of protein)
Expt. 1	
None	1620
$ZnSO_4$ (0·1 mM)	1170
$MnCl_2$ (0·1 mM)	1530
$CuSO_4$ (0·1 mM)	494
$MgCl_2$ (0·1 mM)	1550
$FeSO_4$ (0·1 mM)	730
$FeCl_3$ (0·1 mM)	1570
KF (0·1 mM)	1390
NaSCN (0·1 mM)	1530
$K_3Fe(CN)_6$ (0·1 mM)	1550
NaN_3 (0·1 mM)	1390
Expt. 2	
None	1850
$FeCl_3$ (0·1 mM)	1800
$FeCl_3$ (0·01 mM)	1820
$FeSO_4$ (0·1 mM)	845
$FeSO_4$ (0·01 mM)	1840
$FeSO_4$ (0·1 mM) + pyridoxal phosphate (mM)	1810

and the time-course of formation of δ-aminolaevulate was linear up to 40 min. It was not possible to obtain K_m for this substrate since succinyl-CoA was inhibitory even at comparatively low concentrations (Fig. 3).

Inhibition of δ-aminolaevulate synthetase. The effect of several inhibitors and metal ions on the enzymic formation of δ-aminolaevulate are shown in Table 4. In these experiments EDTA and β-mercaptoethanol were omitted from the reaction mixture.

Special attention was paid to the possible effect of iron on the enzyme, as there is evidence that iron may participate with the enzyme from chicken erythrocytes (Brown, 1958*a*, *b*; Vogel, Richert, Pixley & Schulman, 1960). Ferrous iron was a relatively potent inhibitor of the enzyme prepared from *R. spheroides*, but ferric iron at the same concentration did not have any effect (Table 4). Inhibition by ferrous iron was prevented by adding *o*-phenanthroline or pyridoxal phosphate. Although this indicates that the inhibition was overcome by chelation of the metal, it does not indicate whether the inhibition was initially due to a reaction between iron and pyridoxal phosphate, or whether iron reacted with the protein itself.

Table 5. *Effects of haemin on δ-aminolaevulate synthetase*

The incubation conditions were as indicated in Table 4. The final concentrations of the additions are given in parentheses.

Additions	Specific activity (μm-moles of δ-aminolaevulate/hr./mg. of protein)
Expt. 1	
None (control, no haemin)	1700
Haemin (0·1 μM)	1050
Haemin (1·0 μM)	890
Haemin (10 μM)	760
Haemin (100 μM)	304
Haemin (200 μM)	220
Expt. 2	
Haem control*	990
Haemin control*	930
Haem (100 μM)	210
Haemin (100 μM)	290
Haem (25 μM)	440
Haemin (25 μM)	450

* In these controls a mixture of KOH, HCl and β-mercaptoethanol was added to give the same final concentration as that in the experimental tubes containing the highest concentration of haem or haemin.

Effect of tetrapyrrole derivatives on δ-aminolaevulate synthetase

Protohaemin IX was a relatively potent inhibitor of δ-aminolaevulate synthetase (Table 5). Although the inhibition was pronounced even at very low concentrations of haemin, it was never complete even at the highest concentration examined.

Relative effects of haem and haemin on δ-aminolaevulate-synthetase activity. In most of the experiments on haemin inhibition no attempt was made to control the state of oxidation of the iron atom in the tetrapyrrole nucleus. In all cases haemin (i.e. ferric protoporphyrin) was the compound added to the reaction mixture. However, since β-mercaptoethanol, sometimes present in the reaction mixture, can reduce haemin to haem (ferrous protoporphyrin), it was not clear which of these forms was present in the enzyme reaction mixture. To determine this, haemin was prepared in the usual manner to give a mM stock solution and haem was prepared by reducing the ferric iron in this solution by the addition of a tenfold molar excess of β-mercaptoethanol. All dilutions were made with freshly-boiled double-distilled water and incubation was in sealed tubes under nitrogen. After incubation, pyridine was added to a test sample and a haemochromogen absorption band was observed visually with a hand spectroscope. The results indicate that haemin and haem are essentially equal in their inhibitory power (Table 5).

Table 6. *Haemin inhibition of δ-aminolaevulate synthetase with various concentrations of succinyl-coenzyme A and pyridoxal phosphate*

In Expt. 1 the reaction mixture contained: glycine (100 μmoles), tris buffer, pH 7·8 (50 μmoles), pyridoxal phosphate (0·25 μmole), succinyl-CoA (as shown), haemin (0·01 μmole), enzyme and water to give a final volume of 1·0 ml. In Expt. 2 the reaction mixture contained: glycine (100 μmoles), sodium succinate (100 μmoles), CoA (0·58 μmole), ATP (7·5 μmoles), $MgCl_2$ (10 μmoles), tris buffer, pH 7·8 (50 μmoles), β-mercaptoethanol (1 μmole), EDTA (0·2 μmole), pyridoxal phosphate (as shown), haemin (0·01 μmole, where shown), succinyl-coenzyme A thiokinase (0·1 ml.), enzyme and water to give a final volume of 1·0 ml. In both experiments incubation was for 30 min. at 37°.

Expt. 1			Expt. 2		
	Specific activity (μm-moles of δ-aminolaevulate/hr./mg. of protein)			Specific activity (μm-moles of δ-aminolaevulate/hr./mg. of protein)	
Succinyl-CoA (μmole)	Without haemin	With haemin	Pyridoxal phosphate (μm-moles)	Without haemin	With haemin
0·4	1290	615	360	1445	536
0·2	1390	610	25	1060	490
0·08	627	577	3	635	297

Table 7. *Reversibility of haemin inhibition of δ-aminolaevulate synthetase*

δ-Aminolaevulate synthetase was pretreated for 5 min. at 1° with haemin as shown. The enzyme was then added to the reaction mixture described in Table 4 and incubated for 30 min. at 37°.

Concn. of haemin preincubated with enzyme (μM)	Final concn. of haemin in assay mixture (μM)	Specific activity (μm-moles of δ-aminolaevulate/hr./mg. of protein)
0	0	1800
0	10	910
0	2	1370
10	2	1320
10	1	1640

Table 8. *Effect of various metal complexes of porphyrins on δ-aminolaevulate synthetase*

The incubation conditions were as indicated in Table 4. Each compound was tested at a final concentration of 20 μM. Since the results are from several experiments, results are expressed as percentages of the appropriate control without inhibitor.

Additions to standard assay mixture	Relative enzyme activity (%)
None	100
Protoporphyrin	75
Cobalt–protoporphyrin complex	60
Copper–protoporphyrin complex	66
Zinc–protoporphyrin complex	63
Manganese–protoporphyrin complex	60
Magnesium–protoporphyrin complex	66
Iron–protoporphyrin complex (haemin)	47
Iron–deuteroporphyrin complex	42
Iron–haematoporphyrin complex	46
Iron–coproporphyrin complex	58

Nature of the inhibition by haemin. The inhibition by haemin was examined as a function of substrate concentration to determine whether it was competitive or non-competitive. The inhibition is non-competitive with glycine (Fig. 2). Chemically-prepared succinyl-CoA was used for the examination of inhibition by haemin as a function of succinyl-CoA concentration. There was no competition between haemin and this substrate (Table 6).

The inhibition by haemin was also not influenced by the concentration of pyridoxal phosphate (Table 6).

Reversibility of inhibition by haemin. Enzyme was preincubated for 5 min. at 1° with 10 μM-haemin, then diluted fivefold on addition to the assay mixture. The activity corresponded to that in systems in which 2 μM-haemin had been added directly to the reaction mixture, indicating that the inhibition caused by haemin was reversible (Table 7).

Effect of other tetrapyrroles. The specificity of the inhibition by haemin was examined with protoporphyrin and various metal complexes of porphyrins (Table 8).

Protoporphyrin caused a significant inhibition but the concentration necessary to cause 50% inhibition of the activity was about ten times the haemin concentration required for the same degree of inhibition. The copper complexes and manganese complexes of protoporphyrin inhibited by approximately the same extent as the free tetrapyrrole. The iron complexes of haematoporphyrin, deuteroporphyrin and coproporphyrin all inhibited to a similar extent to haemin. The inhibition caused by iron haematoporphyrin was considerably greater than that found by Burnham (1962*a*). This discrepancy may have been due to variations in the purity of the different batches of haematoporphyrin used to prepare the iron complex; Granick, Bogorad & Jaffe (1953) found it difficult to prepare pure haematoporphyrin free of other porphyrins.

Since the major tetrapyrrole derivative in *R. spheroides* is bacteriochlorophyll this compound, as well as related compounds, was tested. The following were without effect when tested at concentrations between 10 and 100 μM: bacteriochlorophyll, bacteriochlorophyllide, a mixture of chlorophyll *a* and chlorophyll *b*, and a mixture of the corresponding chlorophyllides. Vitamin B_{12} (40 μmM) also had no effect.

Effect of protein-bound haemin. Though there is some evidence that haemin exists to a small extent in the free form, there is little doubt that the greatest part of the haemin within the cell is protein-bound. The effect of some haemoproteins on the activity of δ-aminolaevulate synthetase was therefore examined. Native globin prepared by the method of Rossi-Fanelli *et al.* (1958) was used as a control. Haemoglobin and myoglobin inhibited δ-aminolaevulate synthetase significantly but catalase and cytochrome *c* had no effect (Table 9).

Table 9. *Effect of haemoproteins on δ-aminolaevulate synthetase*

The incubation conditions were as indicated in Table 4. The final concentrations of the additions are given in parentheses.

Additions to standard assay mixture	Specific activity (μm-moles of δ-aminolaevulate/ hr./mg. of protein)
None (control)	1090
Haemin (20 μM)	535
Haemoglobin (32 μM)*	760
Haemoglobin (0·64 μM)*	900
Myoglobin (2·7 μM)*	712
Myoglobin (0·68 μM)*	835
Catalase (0·7 μM)*	1020
Cytochrome *c* (50 μM)*	1060
Globin (60 μM)	1000

* Final concentration in terms of haemin content.

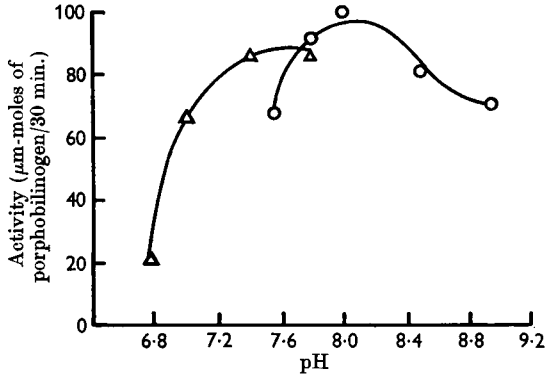

Fig. 4. δ-Aminolaevulate-dehydratase activity as a function of pH. The incubation mixture contained: δ-aminolaevulate (5 μmoles), $MgCl_2$ (5 μmoles), L-cysteine (25 μmoles), buffer (125 μmoles), enzyme (0·1 ml.) and water to give a final volume of 2·5 ml. Incubations were at 37° for 30 min. Buffers: △, potassium phosphate; ○, tris containing K_2SO_4 (40 mM).

Experiments with δ-aminolaevulate dehydratase

The experiments were with the fraction (from the DEAE-cellulose column) that had been purified about 60-fold (Table 3). Under standard assay conditions the amount of porphobilinogen formed was linear with protein between 0·01 and 0·09 mg./ml., and with time for at least 60 min.

Effect of pH *on enzyme activity.* The optimum pH was 7·8 to 8·0 in potassium phosphate buffer, or in tris buffer containing potassium sulphate (40 mM) (Fig. 4).

Requirement for potassium ions. Preliminary experiments with δ-aminolaevulate dehydratase showed that the enzyme was not active in tris buffer. It was active, however, in mixtures of tris and phosphate, indicating that tris was not inhibitory, and suggesting at first a requirement for phosphate. Similar observations were made with the ox-liver enzyme (Gibson *et al.* 1955). Further investigations revealed that the required ion was K^+. This could be provided as the phosphate, sulphate or chloride salt. Sodium sulphate and disodium hydrogen phosphate were without activity (Table 10).

Inhibition studies with δ-aminolaevulate dehydratase. Haemin (20 μM) inhibited the enzyme by only about 23% if added after the enzyme had been preincubated with cysteine (Table 11). If added before preincubation, it inhibited completely.

Previously it has been demonstrated that δ-aminolaevulate dehydratase from ox liver was in-

Table 10. *Effect of potassium ions on δ-aminolaevulate dehydratase*

The incubation mixture contained: δ-aminolaevulate (5 μmoles), L-cysteine (25 μmoles), $MgCl_2$ (5 μmoles), tris buffer, pH 7·8 (160 μmoles), test compounds (final concentrations given in parentheses), enzyme and water to give a final volume of 2·5 ml. Incubation was for 30 min. at 37°. The reaction was stopped with $CuSO_4$, and porphobilinogen was determined.

Additions to standard assay mixture	Specific activity (μm-moles of porphobilinogen/ hr./mg. of protein)
None (control)	650
K_2SO_4 (1·6 mM)	1070
K_2SO_4 (4·8 mM)	2050
K_2SO_4 (16 mM)	3260
K_2SO_4 (40 mM)	4370
KCl (40 mM)	3680
KH_2PO_4–K_2HPO_4 buffer, pH 7·8 (40 mM)	4100
Na_2SO_4 (40 mM)	745

hibited by low concentrations of EDTA (Gibson *et al.* 1955). The enzyme isolated from *R. spheroides* was, however, not inhibited by EDTA (4 mM).

Experiments with intact cells

Effect of haemin on whole cells. The effect of haemin was examined with whole cells synthesizing either free porphyrins or bacteriochlorophyll. In these experiments iron-deficient cells were incubated in the light with glycine and α-oxoglutarate with and without iron citrate under the conditions described by Lascelles (1956). In the absence of added iron, coproporphyrin accumulated with only slight synthesis of bacteriochlorophyll; with iron present, bacteriochlorophyll formation was considerably increased and porphyrins did not accumulate. Haemin at concentrations that inhibit δ-aminolaevulate synthetase caused a considerable decrease in porphyrin formation, but bacteriochlorophyll synthesis (measured with iron added to the incubation system) was much less affected (Table 12). Neither haematohaemin nor protoporphyrin was inhibitory, but deuterohaemin almost completely prevented the formation of both porphyrin and bacteriochlorophyll (Table 12).

Haemin did not inhibit the conversion of δ-aminolaevulate into porphyrin by cell suspensions (Table 12), providing evidence that its effect on porphyrin synthesis from glycine and α-oxoglutarate was confined to its action on δ-aminolaevulate synthetase.

Table 11. *Effect of haemin on δ-aminolaevulate dehydratase*

The incubation mixture contained: δ-aminolaevulate (5 μmoles), potassium phosphate buffer, pH 7·8 (125 μmoles), $MgCl_2$ (5 μmoles), L-cysteine (25 μmoles), haemin (final concentrations shown in parentheses), enzyme and water to give a final volume of 2·5 ml. Incubations were for 30 min. at 37°. The haemin was added with the δ-aminolaevulate after the enzyme had been preincubated for 10 min. in the reaction mixture with cysteine. The reaction was stopped with $CuSO_4$, and porphobilinogen was determined.

Additions to standard assay mixture	Specific activity (μm-moles of porphobilinogen/ hr./mg. of protein)
None (control)	5120
Haemin (8 μM)	4900
Haemin (20 μM)	4420
Haemin (40 μM)	2380

DISCUSSION

R. spheroides was chosen for this work since the controlling effect of iron on porphyrin accumulation is readily demonstrable and also because it contains relatively high concentrations of δ-aminolaevulate synthetase. In micro-organisms other than Athiorhodaceae this enzyme is difficult to detect by the method described above.

Accounts of the purification of this enzyme have appeared (Kikuchi *et al.* 1958; Kikuchi, Kumar & Shemin, 1959), but lack details. The enzyme seems to be labile, and attempts at purification generally

Table 12. *Effect of haemin and other tetrapyrroles on porphyrin and bacteriochlorophyll formation by cell suspensions of* Rhodopseudomonas spheroides

Suspensions of iron-deficient cells (0·6–0·8 mg. dry wt./ml.) were incubated anaerobically in the light in 'mixture I' (containing glycine and α-oxoglutarate) or in 'mixture II' (containing δ-aminolaevulate) under the conditions described by Lascelles (1956). The final concentrations of the added tetrapyrroles are given in parentheses. Porphyrin synthesis was measured in systems containing no added iron, and bacteriochlorophyll synthesis in the presence of 10 μM-iron citrate. Incubation was for 7 hr. in Expts. 1 and 3 and for 8·5 hr. in Expt. 2. N.D., Not determined.

Expt. no.	Substrate mixture	Tetrapyrrole added	Porphyrin formed (μm-moles/ml.)	Bacteriochlorophyll formed (μm-moles/ml.)
1	I	None (control)	31	36
	I	Haemin (5 μM)	16	31
	I	Haemin (10 μM)	17	30
	I	Haemin (20 μM)	6	26
	I	Haemin (40 μM)	3	25
2	I	None (control)	34	56
	I	Haemin (20 μM)	6	47
	I	Protoporphyrin (20 μM)	33	54
	I	Haematohaemin (20 μM)	32	55
	I	Deuterohaemin (20 μM)	2	0
3	I	None	38	N.D.
	I	Haemin (20 μM)	3	N.D.
	II	None	31	N.D.
	II	Haemin (20 μM)	33	N.D.

produce low yields. Some success was attained in the present investigation, possibly as a result of the precautions taken to eliminate inhibitory materials during the fractionation. Preliminary experiments on fractionation with ammonium sulphate were generally unsatisfactory because of poor recovery. This confirmed observations made by Dr K. Gibson (personal communication). It was found, however, that the enzyme could be at least partially protected by carrying out most operations in the presence of β-mercaptoethanol. This. however, was only true if chelating agents, and particularly ones capable of binding ferrous iron, were added before the treatment with ammonium sulphate.

Apart from Cu^{2+}, Fe^{2+} was the most inhibitory metal ion of those tested. The fact that this inhibition can be prevented by using high concentrations of pyridoxal phosphate in the assay mixture suggests that the inhibition is caused by the removal of this cofactor from the system by binding to the metal.

None of the experimental findings indicate that iron is involved in the action of the enzyme, which is contrary to the situation found in avian erythrocytes (Brown, 1958*a*, *b*; Vogel *et al.* 1960). It would seem also that iron is not involved in the synthesis of this enzyme by *R. spheroides*, since it is present in similar amounts in both iron-deficient cells and those cultured with adequate iron.

The nature of the inhibition of the enzyme by haemin supports the possibility that this compound acts as a controlling factor and not simply as an enzyme inactivator. Inhibition was detectable at concentrations as low as 0·1 μM. Even at the highest concentrations tested haemin did not completely inhibit the enzyme activity. This suggests that the inhibition is not the result of a simple stoicheiometric reaction between the haemin and a reactive site on the protein. It also indicates that it is not the result of an interaction with one of the substrates or cofactors. The reversibility of the inhibition caused by haemin adds additional support to the possibility that haemin acts as a controlling factor. Reversibility is a hallmark of negative-feedback systems, according to Umbarger (1961).

It is not necessary to assume that haemin exists free within the cell at any appreciable concentration for it to have a controlling effect on δ-aminolaevulate synthetase, since some haemoproteins were also inhibitory. Work by Banerjee (1962) with haemoglobin and myoglobin, and by Greengard & Feigelson (1962) with tryptophan pyrrolase, indicates that haemin is probably not as tightly bound to some haemoproteins as has been generally assumed. It seems possible, therefore, that sufficient haemin is present within the cells in equilibrium with specific haemoproteins for it to function as a controlling factor.

The experiments with whole cells support the suggestion that haemin exerts a negative-feedback control on porphyrin synthesis. In other systems where a negative-feedback control has been demonstrated, the enzymic step under control has been the first one leading to the end product, and this step is generally energy-consuming and irreversible (Umbarger, 1961). In the present case, haemin inhibits porphyrin synthesis by cell suspensions when glycine and α-oxoglutarate are the starting substrates, i.e. when δ-aminolaevulate synthetase must function. When this step is bypassed by using δ-aminolaevulate as substrate, haemin does not affect the synthesis of porphyrin. The results obtained with inorganic iron, in the present and previous investigations, are compatible with this, if it is assumed that the iron is first converted into a haem compound, which in turn exerts its controlling influence by inhibiting δ-aminolaevulate synthetase.

In whole cells, haemin inhibits porphyrin accumulation much more strongly than it does bacteriochlorophyll formation, indicating that a control exerted by haemin does not appear to interfere with the normal synthesis of bacteriochlorophyll.

It seems incongruous that, in an organism synthesizing two major tetrapyrrole derivatives, haem(in) and bacteriochlorophyll, only haem(in) inhibits the isolated δ-aminolaevulate synthetase. This may have no physiological relevance since bacteriochlorophyll is insoluble in water, and it is doubtful if it ever exists as a free molecule in the cell. The evidence indicates that the later steps in the synthesis of bacteriochlorophyll occur in the chromatophores where it functions. δ-Aminolaevulate synthetase, however, is found in the soluble fraction of the cell. Evidence was sought in extracts of *R. spheroides* for the occurrence of two δ-aminolaevulate synthetases, one for making haemin and the other for making bacteriochlorophyll. An analogous situation has been demonstrated in *Escherichia coli*: two aspartokinases have been shown, each being under the control of a different end product (Stadtman, Cohen, LeBras, De Robichon-Szulmajster, 1961). No evidence for two enzymes was found in the present instance, though it is possible that a δ-aminolaevulate synthetase inside the chromatophore might go undetected. The enzyme is not detectable in other microbial extracts, where it presumably functions since the intact cells synthesize readily detectable amounts of tetrapyrroles (B. F. Burnham & J. Lascelles, unpublished work).

The excretion of porphyrins and porphyrin precursors is sometimes observed in certain diseases of

higher animals, e.g. porphyria in man. Since the excreted porphyrin represents an over-production, one of the systems controlling porphyrin biosynthesis may have broken down, allowing porphyrin and precursors to be formed continuously at the maximum rate. Gerhart & Pardee (1962) have demonstrated that it is possible to alter the active site for the inhibitor on an enzyme without altering the active site for the substrate. Such an enzyme continues to function catalytically, but it is no longer sensitive to inhibitor. It might, therefore, be useful to examine the effect of haemin on enzyme preparations from some of the diseased conditions compared with those from normal animals.

SUMMARY

1. The enzyme, δ-aminolaevulate synthetase, was purified about tenfold from extracts of *Rhodopseudomonas spheroides*.

2. Significant inhibition of δ-aminolaevulate synthetase was caused by 0·1 μM-iron protoporphyrin (haemin); the highest concentration tested (0·2 mM) inhibited the enzyme by about 87%. Other iron porphyrins inhibited to a similar extent, whereas protoporphyrin and other metal complexes of protoporphyrin were less effective. Haemoglobin and myoglobin were also inhibitory.

3. The inhibition by haemin was not competitive with any of the substrates or cofactors, but was reversible by dilution.

4. Porphyrin formation from glycine and α-oxoglutarate by whole-cell suspensions of *R. spheroides* was inhibited by haemin, but conversion of δ-aminolaevulate into porphyrins was unaffected.

5. The characteristics of the inhibition of δ-aminolaevulate synthetase, and the results obtained with whole cells, indicate that one mechanism for control of porphyrin biosynthesis in *R. spheroides* may be through negative feedback by haemin.

6. Succinyl-coenzyme A thiokinase and δ-aminolaevulate dehydratase were purified by about 170- and 70-fold respectively from extracts of *R. spheroides*. δ-Aminolaevulate dehydratase was only slightly inhibited by haemin. It required K^+ ions for activation.

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