Aminolaevulinate Synthetase of *Micrococcus denitrificans*

PURIFICATION AND PROPERTIES OF THE ENZYME, AND THE EFFECT OF GROWTH CONDITIONS ON THE ENZYME ACTIVITY IN CELLS

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1. 5-Aminolaevulinate synthetase was detected in extracts of the non-photosynthetic bacterium *Micrococcus denitrificans*. 2. Activity is high in cells grown anaerobically in a defined nitrate medium, but is low in cells grown in an iron-deficient medium, and in cells grown aerobically. 3. Aminolaevulinate synthetase was purified extensively; it has a molecular weight of about 68000; apparent K_m values for glycine, succinyl-CoA and pyridoxal phosphate are 12mM, 10 μ M and 11 μ M respectively; 2 μ M-haemin and 14 μ M-protoporphyrin inhibit by 50%. 4. The low activity of aminolaevulinate synthetase in iron-deficient cells increases on adding iron salts to cells only under conditions where protein synthesis can occur. 5. In defined nitrate medium with a high Ca^{2+} concentration anaerobic growth yield is higher, but aminolaevulinate synthetase activity is lower than in cells grown in the medium with a low Ca^{2+} concentration. In medium made from AnalaR constituents, growth yield is low and aminolaevulinate synthetase activity is high even in the presence of high concentrations of Ca^{2+} ; on adding Cu^{2+} (0.1 μ M) to the medium growth yield and aminolaevulinate synthetase activity become the same as in non-AnalaR medium. 6. Cells incubated under conditions where protein synthesis does not occur but where electron transport does, lose their aminolaevulinate synthetase activity rapidly. 7. The activities of aminolaevulinate dehydratase and succinic thiokinase do not change under any of the conditions of growth examined. 8. The possible mechanisms controlling aminolaevulinate synthetase activity and the role of this enzyme in controlling the synthesis of haem in this organism are discussed.

5-Aminolaevulinate synthetase catalyses the formation of aminolaevulinate from succinyl-CoA and glycine, which is the first step in the biosynthesis of the porphyrin moiety of haems and chlorophylls and of the corrin ring of vitamin B₁₂ (for review see Burnham, 1969). *Rhodospseudomonas spheroides* and other non-sulphur purple photosynthetic bacteria have a high activity of this enzyme (Burnham, 1970). Crude extract of *Chromatium* strain D, a sulphur purple photosynthetic bacterium, does not show activity, but it can be demonstrated after an extract has been taken through an acetone precipitation step (Burnham, 1970). Activity has been reported in only two non-photosynthetic bacteria, *Propionibacterium shermanii* (Menon & Shemin, 1967) and *Spirillum itersonii* (Clark-Walker *et al.*, 1967). Lascelles (1964) and Burnham (1969) both comment that activity could not be detected in extracts of many other bacteria, but they do not indicate which organisms were tested. Aminolaevulinate synthetase was not extensively purified because of instability of the enzyme from most sources, but the enzyme has now been purified from *R. spheroides* by Warnick &

Burnham (1971) and from rabbit reticulocytes by Aoki *et al.* (1971).

In the present paper the detection of aminolaevulinate synthetase in extracts from *Micrococcus denitrificans* is reported. The enzyme was purified about 800-fold and its properties were studied. The synthetase activity varies markedly under different growth conditions, being barely detectable under some, and the activity decreases rapidly on adding inhibitors of protein synthesis to cultures. The relevance of these findings to the control of porphyrin and haem biosynthesis in this organism is discussed. A preliminary account of some of this work has been presented (Tait, 1972).

Experimental

Chemicals

ATP, CoA and GSH were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; actinomycin D, puromycin, 2-mercaptoethanol and DEAE-cellulose from Sigma (London)

Chemical Co. Ltd., London S.W.6, U.K.; Sephadex G-25, G-100 and G-200 from Pharmacia, Uppsala, Sweden, and bacterial peptone and yeast extract from Oxoid Ltd., London S.E.1, U.K. and from Difco Laboratories, Detroit, Mich., U.S.A. Hydroxyapatite was prepared as described by Tiselius *et al.* (1956) and succinyl-CoA as described by Simon & Shemin (1953).

Methods

Growth of organisms. *Micrococcus denitrificans* was obtained from Professor J. G. Morris, Department of Botany and Microbiology, University College of Wales, Aberystwyth. Organisms were maintained on slopes containing, per litre: bacteriological peptone, 4g; yeast extract, 2g; KH_2PO_4 , 10g; glucose, 10g (sterilized separately); KNO_3 , 20g; agar, 20g; adjusted to pH 6.8 with H_2SO_4 (Scholes & Smith, 1968). Boiling-tubes containing 40ml of this complex medium without agar were inoculated from slopes and grown for 24h at 30°C to provide inocula for the large-scale growth of organisms; 4ml was used for each litre of medium. In most experiments organisms were grown in a defined medium slightly modified from that described by Chang & Morris (1962) with nitrate as sole source of nitrogen. This defined medium contains, per litre: sodium succinate, hexahydrate, 13.5g; K_2HPO_4 , 6.0g; KH_2PO_4 , 4.0g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2g; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 20mg (equivalent to 3.65mg of Ca^{2+} , 91 μM) or less (see below); $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 150mg; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 1mg; KNO_3 , 10.1g. In some experiments nitrate was replaced by NH_4Cl (1.6g/l). After autoclaving the medium and just before inoculating it, 5ml of a sterile iron-citrate solution ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.1g; citric acid monohydrate, 1.05g/l) was added to each litre (final concentration 20 μM). This medium contains less Ca^{2+} than that of Chang & Morris (1962), who added 40mg of CaCl_2 (equivalent to 14.4mg of Ca^{2+} , 360 μM) per litre. In some of the early experiments where 40mg of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (equivalent to 7.3mg of Ca^{2+} , 182 μM) per litre was used, all the components of the medium, except iron-citrate solution, were mixed, water was added to dissolve them, and the resulting solution was autoclaved. The autoclaved medium sometimes contained a precipitate. The Ca^{2+} concentration in the medium affected the extent of growth of the organisms and, more particularly, their content of aminolaevulinic synthetase (see the Results section). In later experiments, medium lacking $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ and $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ was autoclaved and these salts were added as sterile solutions later.

Of the components of the medium all were AnalaR grade except for sodium succinate, K_2HPO_4 and KH_2PO_4 which were 'laboratory-grade reagents'. 'AnalaR' medium was made from AnalaR chemicals; sodium succinate being replaced by AnalaR succinic

acid plus AnalaR NaOH, and K_2HPO_4 by AnalaR Na_2HPO_4 . For a few experiments media were used in which all the components except one were AnalaR grade.

Organisms were grown at 30°C for up to 28h, by which time cultures had entered the stationary phase. For anaerobic growth in medium containing nitrate 1–5-litre conical flasks filled almost to the top were used; N_2 is evolved during anaerobic growth with nitrate. For aerobic growth, flasks were half-full of medium and air was bubbled through the culture, or the flasks were shaken continuously.

After growth, organisms were harvested by centrifugation for 10min at 15000g at 4°C and resuspended in cold 0.05M-Tris-HCl buffer, pH 8.8, previously saturated with N_2 . When organisms were grown on a large scale for purification of aminolaevulinic synthetase, organisms from 10 litres of culture were resuspended to 100ml in buffer, whereas in growth experiments where samples were taken at intervals to follow growth and enzyme activities, cells from 40, 80 or 250ml of culture were resuspended to 2ml in buffer. Suspensions were frozen immediately and kept at -20°C until required.

Preparation of extracts. Suspensions of organisms were disrupted under N_2 in 2–5ml lots by irradiation in an MSE 50W ultrasonic disintegrator operated at maximum output for 1.5min. Suspensions were kept cool by immersing the tube in crushed ice. The suspensions were then centrifuged at 25000g for 15min and the clear yellow supernatants, termed 'enzyme extract', were removed, frozen immediately and kept at -20°C until required.

Determinations. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) as standard. Fractions eluted from columns were monitored for protein by measuring the E_{280} . Growth of cultures was determined by measuring protein content, by the method of Lowry *et al.* (1951), of cells which had been harvested and resuspended in 0.05M-Tris-HCl buffer, pH 8.8. Sometimes the E_{680} of cultures was also measured. DNA content was determined by the diphenylamine method of Dische (1955). Porphyrin excreted into the culture medium was determined as described by Lascelles (1956).

Electrophoresis of proteins on polyacrylamide gel was done at pH 6.6 and 8.4 as described in the *Shandon Instruction Manual* (Shandon Scientific Co. Ltd., London N.W.10, U.K.). Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was done by the method of Shapiro *et al.* (1967) as modified by Weber & Osborn (1969). Electrofocusing of proteins was done on the LKB Electrofocusing column (LKB Instruments Ltd., South Croydon, Surrey, U.K.) over the range pH 2–10. After running for 48h, fractions were collected

and their pH, protein content and enzyme activity were measured.

Enzyme assays. Aminolaevulinate synthetase. The assay mixture contained, in a total volume of 0.5 ml: 20 μ mol of Tris-HCl buffer, pH 7.0; 25 μ mol of glycine; 20 μ mol of sodium succinate; 0.2 μ mol of pyridoxal phosphate; 2 μ mol of ATP (pH 7.0); 3 μ mol of $MgCl_2$; 20 nmol of $MnSO_4$; 40 μ g of CoA; enzyme extract containing up to 0.4 mg of protein. After incubation at 37°C for 1 h, 0.5 ml of 10% (w/v) trichloroacetic acid was added, the precipitated protein was removed by centrifuging, and the aminolaevulinate formed was determined in the supernatant by the method of Mauzerall & Granick (1956). The amount of product formed was proportional to the amount of enzyme extract added up to about 0.4 mg of protein. Activity is expressed as nmol of aminolaevulinate formed/h per mg of protein. Succinyl-CoA, the actual substrate for aminolaevulinate synthetase, is formed rapidly during the assay by succinic thiokinase, whose activity in enzyme extracts is very high (see the Results section). Fractions obtained during the purification of aminolaevulinate synthetase were assayed for activity by adding a partially purified preparation of succinic thiokinase to the assay mixture. This preparation was obtained as a by-product during the purification of aminolaevulinate synthetase (see the Results section). Assays with purified aminolaevulinate synthetase were also done with chemically prepared succinyl-CoA (100 nmol per assay); in these assays only Tris-HCl buffer, glycine, pyridoxal phosphate, succinyl-CoA and enzyme were present and incubation was for 40 min only at 37°C.

Succinic thiokinase (EC 6.2.1.5). The assay mixture contained, in a total volume of 1 ml: 100 μ mol of Tris-HCl buffer, pH 7.7; 100 μ mol of sodium

succinate; 6 μ mol of $MgCl_2$; 4 μ mol of ATP; 50 μ g of CoA; 1.2 mmol of hydroxylamine (0.3 ml of hydroxylamine hydrochloride solution adjusted to pH 7.7 with NaOH); enzyme extract containing up to 1 mg of protein. After incubation at 37°C for 30 min or 1 h, the succinohydroxamate formed was determined by the method of Lipmann & Tuttle (1945). Activity is expressed as nmol of succinohydroxamate formed/h per mg of protein.

Aminolaevulinate dehydratase (EC 4.2.1.24). The assay mixture contained, in a total volume of 2 ml: 125 μ mol of phosphate buffer (KH_2PO_4 -NaOH), pH 7.5; 5 μ mol of $MgCl_2$; 5 μ mol of aminolaevulinate; enzyme extract containing up to 1 mg of protein. After incubation at 37°C for 30 min or 1 h, 0.1 ml of $CuSO_4 \cdot 5H_2O$ (20%, w/v) was added and the suspension was centrifuged. The amount of porphobilinogen formed was determined in the supernatant by the method of Mauzerall & Granick (1956). Activity is expressed as nmol of porphobilinogen formed/h per mg of protein.

Results

Activities of enzymes in extracts from cells grown in different ways

The activities of aminolaevulinate synthetase and dehydratase and succinic thiokinase were measured in enzyme extracts from cells grown anaerobically and aerobically in different media (Table 1). Aminolaevulinate synthetase activity was high only in cells grown anaerobically in defined nitrate-containing medium with sufficient iron. Organisms grown anaerobically in complex nitrate medium had an intermediate activity. This activity, but not that in

Table 1. *Enzyme activities in M. denitrificans grown in different ways*

Cultures were grown at 30°C for 17–24 h in the media and under the conditions stated. All the defined media contained 40 mg of $CaCl_2 \cdot 6H_2O/l$, and the same batches of non-AnalaR sodium succinate, KH_2PO_4 and K_2HPO_4 . The medium with 'low iron' contained 0.4 μ M instead of the usual concentration of 20 μ M. Enzyme extracts were prepared and enzymes were assayed as described in the Experimental section. Where two values for enzyme activities are given, they are the results from two separate growth experiments.

Growth conditions	Medium	Enzyme activities (nmol of product/h per mg of protein)		
		Aminolaevulinate synthetase	Aminolaevulinate dehydratase	Succinic thiokinase
Anaerobic	Defined nitrate	55, 74	45, 43	6900, 5470
Anaerobic	Defined nitrate-low iron	13, 7	46, 39	14400, 5000
Anaerobic	Complex nitrate	22	44	10000
Aerobic	Defined nitrate	6, 13	52, 37	7500, 5950
Aerobic	Defined ammonia	12, 16	39, 29	5300, 3740

enzyme extracts from cells grown under the other conditions, was increased by a factor of about two by assaying in the presence of 50–300 μM -cysteine, GSH or 2-mercaptoethanol. The activity of aminolaevulinate dehydratase, which is of the same order of magnitude as that of aminolaevulinate synthetase, and that of succinic thiokinase, which is two orders of magnitude higher, were not markedly affected by the conditions of growth. These three enzymes are 'soluble'; the activities per ml of extract are the same in sonicated preparations before centrifuging as in supernatants obtained after centrifuging at 100000g for 1 h. Aminolaevulinate dehydratase activity was low or undetectable in enzyme extracts which contained less than about 5 mg of protein/ml, that is in enzyme extracts prepared by sonicating relatively dilute cell suspensions. Activity could not be restored by doing the assay in the presence of cysteine, GSH or 2-mercaptoethanol, or by preincubation with these compounds before assay. The reason for the low activity is not known, but it is not related to the way in which the cells are grown. Enzyme extracts with a higher protein content prepared from the same cells exhibited high aminolaevulinate dehydratase activity. The specific activities of aminolaevulinate synthetase and of succinic thiokinase were the same in enzyme extracts with low and high protein content.

At the end of anaerobic, but not aerobic, growth in defined nitrate medium cultures of *M. denitrificans* excreted small amounts of porphyrin into the medium. The culture supernatants fluoresced pink when viewed under a Wood's lamp. The porphyrin was extracted from the culture supernatant and found to be coproporphyrin as judged by its spectra in acid and in ethyl acetate. The amounts found were about 2–4 nmol/ml in cultures grown in medium with a high iron concentration, and 1–2 nmol/ml in cultures grown without added iron.

All the work described below was done with organisms grown anaerobically in a defined medium containing nitrate.

Purification of aminolaevulinate synthetase

The purification was started with organisms from 40 litres of culture grown anaerobically in 5-litre lots in defined nitrate medium. All operations were carried out at 0–4°C, and all buffers contained 2 mM-2-mercaptoethanol, which was essential for stabilizing the enzyme during the later stages of purification.

The suspension of organisms (400 ml) was sonicated in 5 ml lots and then centrifuged at 100000g for 90 min. To the supernatant (330 ml) 82.5 ml of 2% (w/v) protamine sulphate was added slowly with stirring. After 20 min the suspension was centrifuged at 25000g for 10 min. The supernatant (386 ml) was adjusted to 38% saturation with solid $(\text{NH}_4)_2\text{SO}_4$

(88.9 g) and, after stirring for 20 min, the suspension was centrifuged at 25000g for 15 min and the supernatant was removed. Further addition of $(\text{NH}_4)_2\text{SO}_4$ to this supernatant yielded fractions precipitated at 50% saturation and 60% saturation that contained the bulk of the aminolaevulinate dehydratase and succinic thiokinase activities respectively. The protein precipitating between 50 and 60% saturation, which is free of aminolaevulinate synthetase activity, was redissolved in a small volume of buffer and used as the preparation of succinic thiokinase in assays of aminolaevulinate synthetase activity. The protein precipitated at 38% saturation was redissolved in 0.5 M-Tris-HCl buffer, pH 7.7, to a volume of 25 ml and chromatographed on a column (54 cm $\times$ 3.2 cm) of Sephadex G-200. Elution was performed with the same buffer at a rate of 15 ml/h. Fractions (4.5 ml) were collected and their aminolaevulinate synthetase activity and E_{280} measured. Fractions with high specific activity (nmol of aminolaevulinate formed/h per unit of E_{280}) were pooled (fractions 50–65), the pH was adjusted to 8.8 and the solution was applied to a column (7.5 cm $\times$ 2 cm) of DEAE-cellulose equilibrated with 0.05 M-Tris-HCl buffer, pH 8.8. Elution was performed with a linear gradient made from 200 ml each of 0.05 M-NaCl–0.05 M-Tris-HCl buffer, pH 8.8, and 0.225 M-NaCl–0.05 M-Tris-HCl

Fig. 1. Chromatography of aminolaevulinate synthetase on Sephadex G-100

A sample (4 ml) of partially purified aminolaevulinate synthetase (see the text) was applied to a column (60 cm $\times$ 2.25 cm) of Sephadex G-100. Elution was with 0.05 M-Tris-HCl buffer, pH 7.7, 2 mM-mercaptoethanol and 4.5 ml fractions were collected. The E_{280} (○) was measured in cuvettes with a 1 cm light-path and portions were assayed for aminolaevulinate synthetase activity (●). There was no enzyme activity or protein in fractions 0–15.

buffer, pH 8.8. Fractions (4.5 ml) with high specific activity (fractions 41–50) were pooled. The solution (45 ml) was concentrated to about 2 ml in an Amicon Ultrafiltration cell with a UM-10 membrane. The concentrated solution was diluted with 40 ml of 1 mM-phosphate buffer (KH_2PO_4 –NaOH), pH 6.9, and re-concentrated to 2 ml. The solution was diluted to 20 ml with the same buffer and applied to a column (3 cm $\times$ 2 cm) of hydroxyapatite previously equilibrated with this buffer at an elution rate of about 10 ml/h. Elution was performed first with 30 ml of the 1 mM-phosphate buffer, pH 6.9, and then with a linear gradient made from 100 ml each of 1 mM-phosphate buffer, pH 6.9, and 40 mM-phosphate buffer, pH 6.9. Fractions (4.5 ml) were collected and those with high specific activity (fractions 35–46) were pooled and applied to a column (2.5 cm $\times$ 1 cm) of DEAE-cellulose equilibrated with 0.05 M-Tris–HCl buffer, pH 7.7. The enzyme was eluted with 0.25 M-NaCl–0.05 M-Tris–HCl buffer, pH 7.7, and the solution (4 ml) was applied to a column (60 cm $\times$ 2.25 cm) of Sephadex G-100 equilibrated with 0.05 M-Tris–HCl buffer, pH 7.7. The column was eluted with the same buffer and fractions (4.5 ml) were collected. Aminolaevulinate synthetase activity was eluted just before the peak of protein (Fig. 1). Fractions 21–23, which had the highest specific activity, were pooled and concentrated by adsorption on to DEAE-cellulose and elution with 0.25 M-NaCl–0.05 M-Tris–HCl buffer, pH 7.7. This final enzyme solution (3 ml) was frozen in 0.3 ml portions and kept at -20°C until required.

By this procedure (Table 2) the aminolaevulinate synthetase was purified about 800-fold. A number of different purifications were done in this way. The amount of $(\text{NH}_4)_2\text{SO}_4$ required to precipitate the bulk of the aminolaevulinate synthetase activity varied between 38 and 40% saturation and it was convenient to do this step on a small scale first with each preparation of protamine sulphate super-

natant. The hydroxyapatite chromatography was not reproducible if the rate of elution of the column was too high.

No increase in total activity occurs at any step during the purification. When enzyme extract from cells grown anaerobically in complex nitrate medium was purified, more than 100% yield of enzyme was obtained at the $(\text{NH}_4)_2\text{SO}_4$ step. As shown above, the aminolaevulinate synthetase activity of enzyme extract from cells grown in this way was increased by a factor of about two by assaying in the presence of cysteine.

Properties of purified aminolaevulinate synthetase

Although it is clear from Fig. 1 that the enzyme has not been purified to homogeneity, the final preparation of enzyme showed only one protein band on disc-gel electrophoresis at pH 6.6 and at 8.4. The Sephadex G-100 column used in the final step of purification was calibrated by chromatography of Blue Dextran and a number of proteins of known molecular weight: bovine plasma albumin (mol.wt. 66500), ovalbumin (mol.wt. 45000; its dimer was also detected) and cytochrome *c* (mol.wt. 12500). A plot of log mol.wt. against elution volume divided by void volume gave a straight line, and aminolaevulinate synthetase had a molecular weight of approx. 68000. The final preparation of aminolaevulinate synthetase was treated with sodium dodecyl sulphate and 2-mercaptoethanol and subjected to electrophoresis. Two protein bands were obtained, one with the same mobility as bovine albumin that is probably aminolaevulinate synthetase, and the other with a mobility slightly less than that of ovalbumin that is probably the impurity eluted from the Sephadex G-100 column just after aminolaevulinate synthetase. From the relative intensities of staining of the bands on electrophoresis in sodium dodecyl sulphate the ratio of aminolaevulinate synthetase to contaminating protein

Table 2. *Purification of aminolaevulinate synthetase*

Fraction	For details see the text.			
	Total activity (nmol of aminolaevulinate/h)	Total protein (mg)	Specific activity (nmol of aminolaevulinate/h per mg of protein)	Purification factor
Crude sonicate	313000	6600	47	1
100000g supernatant	313000	4420	71	1.5
Protamine sulphate supernatant	294000	3305	89	1.9
0–38% $(\text{NH}_4)_2\text{SO}_4$ fraction	244000	388	630	13.4
Sephadex G-200 eluate	157730	116	1540	32.9
DEAE-cellulose eluate	55000	13.5	4070	86.2
Hydroxyapatite eluate	29610	1.2	25000	532
Sephadex G-100 eluate	11080	0.3	37000	788

Fig. 2.

Table 3. *Effect of Ca²⁺ on anaerobic growth and aminolaevulinate synthetase activity in non-AnalaR defined nitrate medium*

Ca²⁺ and Mn²⁺ (as stated) and iron-citrate (20 µmol/l) were added to sterile non-AnalaR defined nitrate medium. Cultures were grown anaerobically and portions were removed at the times stated for estimation of growth and aminolaevulinate synthetase activity.

Expt.	Ca ²⁺ (µM)	Mn ²⁺ (µM)	Growth (mg of protein/ml of culture)				Aminolaevulinate synthetase activity (nmol of aminolaevulinate/h per mg of protein)			
			16.5h	19h	21.5h	24h	16.5h	19h	21.5h	24h
1	18	4.5	0.30	0.38	0.43	0.46	34	35	55	61
	46	4.5	0.32	0.50	0.59	0.62	35	29	51	47
	91	4.5	0.33	0.56	0.62	0.66	34	25	43	51
	182	4.5	0.30	0.53	0.67	0.79	33	17	20	21
2		Time ...	17h	19.5h	21.5h	24h	17h	19.5h	21.5h	24h
	46	1.1	0.39	0.64	0.70	0.65	37	37	64	22
	182	4.5	0.42	0.60	0.73	0.70	19	19	24	6
3		Time ...	18h	20h	22h	24h	18h	20h	22h	24h
	4.5	0.9	0.45	0.50	0.55	0.61	43	64	77	60
	9	0.9	0.43	0.50	0.56	0.59	40	52	89	70
	18	0.9	0.56	0.65	0.73	0.74	43	56	57	43
	36	0.9	0.58	0.74	0.78	0.82	33	36	34	12
4		Time ...		20h	22h	24h		20h	22h	24h
	—	—		0.29	0.34	0.40		46	46	46
	—	0.9		0.27	0.33	0.38		46	38	45
	4.5	—		0.34	0.42	0.47		45	58	58
	4.5	0.9		0.40	0.46	0.50		52	79	69

is about 3:7. Electrofocusing of a partially purified preparation of aminolaevulinate synthetase showed that it had an isoelectric point of 4.9.

With the purified enzyme, apparent K_m values of about 11 µM for pyridoxal phosphate, 12 mM for glycine and 10 µM for succinyl-CoA were found. There was no activity in the absence of added pyridoxal phosphate. With succinyl-CoA as substrate the pH optimum was about 7.0 in 0.1 M-phosphate buffer (KH₂PO₄–NaOH) and about 7.5 in 0.1 M-Tris–HCl buffer; the activity changed little with pH and it was about the same in both buffers. In 0.2 M-phosphate buffer the activity was the same as in the 0.1 M-phosphate buffer, but 0.2 M-Tris–HCl buffer inhibited the activity by about 50%. No aminoketone was formed when acetyl-CoA was used instead of succinyl-CoA. Haemin (2 µM) caused a 50% inhibition of activity in assays done with succinyl-CoA; protoporphyrin at 14 µM also caused 50% inhibition. In

assays with succinate, CoA, ATP and succinic thiokinase both with the purified enzyme and with the original enzyme extract 6 µM-haemin was required to produce 50% inhibition. Even with the highest concentration of haemin tested (30 µM), succinic thiokinase activity was not affected. 1,10-Phenanthroline (1 mM) inhibited activity of the purified enzyme by about 50%, but EDTA (2.5 mM), sodium azide (4 mM), αα'-bipyridyl (4 mM) and 8-hydroxyquinoline (4 mM) had little or no inhibitory effect.

Effects of Ca²⁺ and Cu²⁺ on growth and on aminolaevulinate synthetase activity

Initially, with defined nitrate medium, different batches of cells grew anaerobically to different extents and their aminolaevulinate synthetase activities were different. The results obtained were either as in

Fig. 2. *Growth of M. denitrificans anaerobically in defined nitrate medium*

Organisms were grown anaerobically in 5-litre flasks in defined nitrate medium containing 40 mg of CaCl₂·6H₂O/l, which was added before autoclaving the medium. At the times stated, samples were taken and used to measure growth as mg of protein/ml of culture (○, ●) and by E_{680} (Δ, ▲), and aminolaevulinate synthetase activity (□, ■). Two 5-litre flasks were used in each experiment as represented by the open and closed symbols. Different batches of medium were used for the two experiments illustrated in (a) and (b) respectively.

Fig. 2(a) or as in Fig. 2(b). These two experiments were done within a week of each other with medium made up fresh for each experiment. In experiment (a), the culture reached the stationary phase at a lower level of growth than in experiment (b). The aminolaevulinate synthetase activity was high in experiment (a) and only decreased a little during the stationary phase, whereas in experiment (b) the activity was low even during growth and it fell to a very low value at the end of growth. Culture (a) remained colourless throughout, whereas culture (b) became yellow towards the end of growth, the yellow colour being in the medium and not in the cells. The increase of DNA content in the cultures followed closely the increases in protein content and E_{680} . Aminolaevulinate dehydratase and succinic thiokinase activities of both cultures were about the same at all stages of growth and neither activity decreased even on incubating cultures for a further 24h. The activity of aminolaevulinate synthetase in culture (b) at the end of growth was just above the limit of detection of the assay method. Indeed, if such extracts had been tested without the knowledge that cells had contained activity at an earlier stage of growth, the low value obtained might have been considered to be insignificant. However, by doing suitable controls it was clear that a low activity was still present.

Most of the experiments gave results similar to those described in Fig. 2(a), but with those batches of medium giving results similar to those in Fig. 2(b) the Ca^{2+} content affected both growth and

aminolaevulinate synthetase activity (Table 3). With small amounts of added Ca^{2+} results like those in Fig. 2(a) were obtained and, with larger amounts of Ca^{2+} , results like those in Fig. 2(b) were obtained. Growth increased as the amount of Ca^{2+} added to the medium was increased, and with $182\mu\text{M}$ - Ca^{2+} the culture became yellow at the end of growth. With low concentrations of Ca^{2+} the aminolaevulinate synthetase activity increased towards the end of growth and remained high, or fell only slightly, in the stationary phase. With high concentrations of Ca^{2+} activity was low at early stages of growth and fell even further at the end of growth. With intermediate concentrations of Ca^{2+} there were slight variations from experiment to experiment; in some aminolaevulinate synthetase activity was high at the end of growth and then it fell rapidly. The activity of aminolaevulinate dehydratase and of succinic thiokinase were not affected by growing the cell with different amounts of Ca^{2+} . Medium without added Ca^{2+} contained about 0.5mg of Ca^{2+} /litre ($12.5\mu\text{M}$) as measured by the method of Kuttner & Cohen (1927).

Cells grown in medium containing $182\mu\text{M}$ - Ca^{2+} with twice the usual amount of succinate, or Mg^{2+} , or SO_4^{2-} or MoO_4^{2-} , or up to four times the amount of iron-citrate, had low aminolaevulinate synthetase activity and cultures were yellow at the end of growth. With twice the amount of KNO_3 the culture behaved as if less Ca^{2+} were present; aminolaevulinate synthetase activity was high at the end of growth and then

Table 4. *Effect of Ca^{2+} on anaerobic growth and aminolaevulinate synthetase activity in AnalaR defined nitrate medium*

Ca^{2+} at the concentrations shown, Mn^{2+} ($4.5\mu\text{mol/l}$) and iron-citrate ($20\mu\text{mol/l}$) were added to sterile AnalaR defined nitrate medium. Cultures were grown anaerobically and portions were taken at the times stated for estimating growth and aminolaevulinate synthetase activity.

Expt.	Ca^{2+} (μM)	Growth (mg of protein/ml of culture)				Aminolaevulinate synthetase activity (nmol of aminolaevulinate/h per mg of protein)			
	Time ...	0h	22h	24h	26h	20h	22h	24h	26h
1	None	0.21	0.24	0.28	—*	25	36	45	—
	4.5	0.20	0.23	0.27	—	32	40	47	—
	9	0.22	0.23	0.27	—	33	40	52	—
	18	0.22	0.25	0.30	—	32	48	57	—
	36	0.26	0.28	0.34	0.34	41	45	60	64
	72	0.29	0.33	0.34	0.39	43	51	61	64
	128	0.35	0.37	0.41	0.43	46	58	61	53
	182	0.39	0.39	0.40	0.45	50	53	51	41
2	182	—	—	0.33	0.36	—	—	49	49
	364	—	—	0.33	0.36	—	—	38	37
	728	—	—	0.37	0.40	—	—	28	27

* Not tested.

it fell rapidly. Addition of Ca^{2+} ($182\text{ }\mu\text{mol/l}$), with or without iron-citrate, to a culture grown to 0.42 mg of protein/ml of culture in medium containing $9\text{ }\mu\text{mol}$ of Ca^{2+}/l did not cause growth to increase or aminolaevulinate synthetase activity to fall. Addition of Ca^{2+} to an identical culture earlier in the growth (0.29 mg of protein/ml of culture) caused the culture to grow more than the control; aminolaevulinate synthetase activity decreased and the culture became yellow.

With the same range of Ca^{2+} concentrations, growth in AnalaR medium (Table 4) was less than in non-AnalaR medium (cf. Table 3). Even with $182\text{ }\mu\text{M-Ca}^{2+}$ cultures were colourless and had high aminolaevulinate synthetase activities. Cultures grown with $364\text{ }\mu\text{M-}$ and $728\text{ }\mu\text{M-Ca}^{2+}$ were yellow and had low aminolaevulinate synthetase activities, but their growth was still not as high nor their aminolaevulinate synthetase activity as low as those of cultures in non-AnalaR medium (compare Tables 3 and 4).

Since the amounts of Ca^{2+} present as contaminant in non-AnalaR medium (about $12.5\text{ }\mu\text{mol/l}$) and AnalaR medium (about $10\text{ }\mu\text{mol/l}$) were similar, it seemed that the different responses to excess of Ca^{2+} involved some additional factor. This factor was present in reagent-grade K_2HPO_4 and sodium succinate, but not in KH_2PO_4 . Growth of cultures in medium containing either K_2HPO_4 or sodium succinate as the only non-AnalaR component was the same as in non-AnalaR medium, and aminolaevulinate synthetase activity was very low. Addition of Zn^{2+} ($2\text{--}40\text{ }\mu\text{M}$) to AnalaR medium containing high Ca^{2+} concentrations ($182\text{ }\mu\text{M}$) did not increase growth, but concentrations of $16\text{ }\mu\text{M}$ and above did cause the culture to become yellow and also decreased the aminolaevulinate synthetase activity. Addition of Cu^{2+} to AnalaR medium had marked effects on

growth and aminolaevulinate synthetase activity (Table 5). With low Ca^{2+} concentrations ($9\text{ }\mu\text{M}$), $0.04\text{ }\mu\text{M-Cu}^{2+}$ increased growth slightly, $0.1\text{ }\mu\text{M}$ was more effective and $0.3\text{ }\mu\text{M}$ caused the culture to grow to the same extent as in non-AnalaR medium with high Ca^{2+} concentrations. The aminolaevulinate synthetase activities were about the same with $0.04\text{ }\mu\text{M-}$ and $0.1\text{ }\mu\text{M-Cu}^{2+}$ as in its absence, but with $0.3\text{ }\mu\text{M-Cu}^{2+}$ the activity fell markedly towards the end of growth. With high Ca^{2+} concentrations ($182\text{ }\mu\text{M}$) $0.04\text{ }\mu\text{M-Cu}^{2+}$ increased growth slightly, whereas $0.1\text{ }\mu\text{M-}$ and $0.3\text{ }\mu\text{M-Cu}^{2+}$ increased it to the level obtained with non-AnalaR medium and cultures were yellow. With $0.04\text{ }\mu\text{M-Cu}^{2+}$, aminolaevulinate synthetase activity decreased at the end of growth whereas with higher concentrations of Cu^{2+} very low activity was found. Zn^{2+} alone or in the presence of Cu^{2+} had little effect on growth or on aminolaevulinate synthetase activity. Organisms did not grow in AnalaR medium containing $0.5\text{ }\mu\text{M-Cu}^{2+}$. When a batch of non-AnalaR medium which allowed high growth of cells was extracted with a solution of diphenylthiocarbazone in carbon tetrachloride to remove trace metals growth was much lower; addition of Cu^{2+} ($0.1\text{ }\mu\text{M}$) to this extracted medium restored growth to its former level.

Aminolaevulinate synthetase activities in sonicated samples before centrifuging and in enzyme extracts were not affected by preincubating or assaying with EDTA, Fe^{2+} , Fe^{3+} or reducing agents at a range of concentrations. Preincubation of enzyme extract (0.4 mg of protein in 0.1 ml) with $20\text{ }\mu\text{M-}$, $45\text{ }\mu\text{M-}$ and $84\text{ }\mu\text{M-Cu}^{2+}$ at 37°C for 15 min before assaying inactivated it by 16 , 47 and 68% respectively. Additions of these concentrations of Cu^{2+} to enzyme extract, just before adding substrates and assaying, inactivated the enzyme by 15 , 17 and 32% respectively. When

Table 5. *Effects of Cu^{2+} on anaerobic growth and aminolaevulinate synthetase activity in AnalaR defined nitrate medium*

Ca^{2+} and Cu^{2+} at the concentrations shown, Mn^{2+} ($4.5\text{ }\mu\text{mol/l}$) and iron-citrate ($20\text{ }\mu\text{mol/l}$) were added to sterile AnalaR defined nitrate medium. Cultures were grown anaerobically and portions were taken at the times stated for estimation of growth and aminolaevulinate synthetase activity.

Ca ²⁺ (μM)	Cu ²⁺ (μM)	Growth (mg of protein/ml of culture)			Aminolaevulinate synthetase activity (nmol of aminolaevulinate/h per mg of protein)			
		Time ...	22h	24h	26h	22h	24h	26h
9	—		0.21	0.26	0.27	28	27	36
9	0.04		0.26	0.30	0.34	31	39	50
9	0.10		0.38	0.40	0.43	36	44	43
9	0.30		0.52	0.66	0.76	22	28	6
182	—		0.34	0.37	0.39	36	44	44
182	0.04		0.46	0.51	0.51	36	36	22
182	0.10		0.71	0.69	0.70	10	7	5
182	0.30		0.56	0.65	0.70	12	10	7

Table 6. *Effect of iron on anaerobic growth and aminolaevulinate synthetase activity in non-AnalaR defined nitrate medium*

Defined nitrate medium containing $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (40 mg/l) was autoclaved and iron-citrate was added as stated. Organisms were grown anaerobically and samples were taken at 23 h in Expt. 1 and at 24 h in Expt. 2 for measuring growth and aminolaevulinate synthetase activity.

Iron (μM)	Growth (mg of protein/ml of culture)		Aminolaevulinate synthetase activity (nmol of aminolaevulinate/h per mg of protein)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
None	0.08	0.05	5	20
0.4	0.22	0.20	8	13
1.6	0.32	0.37	21	13
2.4	—*	0.40	—	30
4.0	0.35	0.51	48	20
20.0	0.48	0.53	50	71
40.0	0.49	—	48	—

* Not tested.

extracts with high and low enzyme activities were mixed and assayed before and after preincubation in the absence of substrates, the enzyme activity was the sum of the two separate enzyme activities. This shows that there is no activator in extracts with high enzyme activity and no inhibitor in extracts with low enzyme activity. Removal of small molecules by passing enzyme extracts through Sephadex G-25 had no effect on enzyme activities of extracts. Purification of an extract with low enzyme activity was done as described above in the presence of 2 mM-mercapto-ethanol and the active fractions obtained from the DEAE-cellulose column were pooled. The yields and the extent of purification at each step were about the same as in a purification with an extract with high enzyme activity. This shows that if an inhibitor is present in extracts with low enzyme activity, it must be tightly bound to the enzyme.

Aminolaevulinate synthetase activity in iron-deficient cells

Organisms were grown anaerobically in defined nitrate medium with different amounts of added iron-citrate (Table 6). Iron deficiency decreased the growth of cultures, and the cells had a very low content of aminolaevulinate synthetase. The specific activities of aminolaevulinate dehydratase and succinic thio-kinase were the same in all cells. Addition of iron to an iron-deficient culture towards the end of growth (Fig. 3*a*) caused a marked increase in the activity of aminolaevulinate synthetase, but had little effect on growth. This experiment was repeated a number of times. In some experiments a different response to iron was obtained (Fig. 3*b*). Cells started to grow

rapidly after addition of iron and the aminolaevulinate synthetase activity increased and then decreased. Cultures grown in the same batch of medium as used in Fig. 3*b*, but with a high iron concentration from the start, had the characteristics shown in Fig. 2*b*. These differences are probably due to the different amounts of Cu^{2+} in the medium (see above).

The aminolaevulinate synthetase activity did not increase on adding iron to iron-deficient cells when chloramphenicol (18 mg/l) was added at the same time (Fig. 4) and a smaller amount of chloramphenicol (6 mg/l) delayed the response to iron. Addition of acriflavin (10 mg/l) at the same time as iron also abolished the increase in aminolaevulinate synthetase activity. Actinomycin D (1 mg/l) had no effect. Chloramphenicol did not inhibit activity when added directly to the assay mixture. There was no increase in aminolaevulinate synthetase activity when cells grown in a low concentration of iron were resuspended in N_2 -saturated 0.05 M-phosphate buffer (KH_2PO_4 -NaOH), pH 7.5, and iron was added.

Changes in aminolaevulinate synthetase activity under other growth and incubation conditions

Most of the experiments reported in this section were done with batches of non-AnalaR medium, which gave low growth and high aminolaevulinate synthetase activity even with a high concentration of Ca^{2+} , i.e. in medium with a low Cu^{2+} content. Identical results were obtained with batches of non-AnalaR medium which gave low growth and high aminolaevulinate synthetase activity only when a low concentration of Ca^{2+} was present.

Addition of chloramphenicol (18 mg/l) and of

Fig. 3. Effect on aminolaevulinate synthetase activity of adding iron to an iron-deficient culture

Two cultures were grown for 24 h in a defined nitrate medium containing 40 mg of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ and $2.4 \mu\text{mol}$ of iron-citrate/l. At 24 h, $4 \mu\text{mol}$ of iron-citrate/l was added to one flask (●, ■); none was added to the other flask (○, □). Samples were taken at intervals to measure growth (□, ■) and aminolaevulinate synthetase activity (○, ●). Different batches of medium were used for the two experiments illustrated in (a) and (b).

Fig. 4. Effect on aminolaevulinate synthetase activity of adding iron and chloramphenicol to an iron-deficient culture

Three cultures were grown for 17 h in defined nitrate medium containing 40 mg of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ and $2.4 \mu\text{mol}$ of iron-citrate/l. At 17 h, $4 \mu\text{mol}$ of iron-citrate/l was added to each culture, and chloramphenicol was also added at the concentrations as follows: none (●); 6 mg/l (○); 18 mg/l (■). Samples were taken at the times shown to measure aminolaevulinate synthetase activity.

Fig. 5. Effect on aminolaevulinate synthetase activity of adding chloramphenicol and puromycin

Three cultures were grown anaerobically in defined nitrate medium to which $9 \mu\text{mol}$ of Ca^{2+} /l, $0.9 \mu\text{mol}$ of Mn^{2+} /l and $20 \mu\text{M}$ -iron-citrate had been added after autoclaving. After they had been growing for 22 h, chloramphenicol (18 mg/l, ●) was added to one, and puromycin (8 mg/l, ○) was added to another; no addition was made to the third flask (□). Samples were taken at intervals to measure aminolaevulinate synthetase activity.

puromycin (10mg/l) to cultures growing in medium with a high iron concentration caused a rapid loss of aminolaevulinate synthetase activity (Fig. 5). About one-half the activity was lost in 1 h. The same rapid loss of activity was caused by acriflavin (5mg/l) or by bubbling air through the culture. The loss of activity with chloramphenicol was not prevented by prior addition of phenylmethane sulphonyl fluoride (300mg/l). This compound, which is a specific inhibitor of 'serine proteases', inhibits protein breakdown in *Escherichia coli* A33 deprived of glucose (Goldberg, 1971a). Chloramphenicol and puromycin did not cause any decrease in aminolaevulinate synthetase activity when added to a culture a short time after NaCN (5mM) or NaN₃ (10mM), or if they were added to organisms that had been harvested and resuspended in N₂-saturated 0.05M-phosphate buffer (KH₂PO₄-NaOH), pH 7.5. Cells suspended in buffer retained their aminolaevulinate synthetase activity for up to 2 h, but activity fell rapidly on aeration or on adding KNO₃ (10.1 g/l), or KNO₃ plus sodium succinate (13.5 g/l), but not sodium succinate alone. The effect of KNO₃ was blocked by N₃⁻ and by CN⁻, but the effect of air was blocked only by CN⁻.

Aminolaevulinate dehydratase and succinic thiokinase activities did not change under any of these conditions.

Discussion

Properties of aminolaevulinate synthetase

The enzyme from *M. denitrificans* has a molecular weight of about 68000, which is close to the value of 57000 found for the enzyme from *R. sphaeroides* (Warnick & Burnham, 1971); both enzymes appear to consist of a single polypeptide chain. The enzyme from rat liver mitochondria, mol.wt. 115000 (Hayashi *et al.*, 1970), is larger and the one from rabbit reticulocytes, mol.wt. 200000 (Aoki *et al.*, 1971), is larger still. The apparent K_m values for pyridoxal phosphate, succinyl-CoA and glycine, and the pH optima of the enzymes from *M. denitrificans*, *R. sphaeroides* (Warnick & Burnham, 1971; Yubisui & Yoneyama, 1972) and rabbit reticulocytes (Aoki *et al.*, 1971) are similar. These enzymes, in common with aminolaevulinate synthetases from most sources, are markedly inhibited by low concentrations of haemin and by slightly higher concentrations of protoporphyrin. By contrast, the aminolaevulinate synthetase from *S. itersonii* is relatively insensitive to inhibition by haemin (Ho & Lascelles, 1971). The inhibition by 1,10-phenanthroline may indicate that the aminolaevulinate synthetase from *M. denitrificans* contains a metal. However, the high concentration required to produce inhibition, and the lack of effect of other

chelating agents, suggests that the effect of 1,10-phenanthroline may not be due to its binding a metal.

The specific activity of the purified enzyme from *R. sphaeroides* is 130000nmol of aminolaevulinate/h per mg of protein (Warnick & Burnham, 1971), that of the enzyme from *M. denitrificans*, which is not completely pure, is 37000, whereas that of the enzyme from rabbit reticulocytes is only 312 (Aoki *et al.*, 1971). It will be of interest to see whether other mammalian aminolaevulinate synthetases also have low specific activities.

Role of aminolaevulinate synthetase in controlling the biosynthesis of haem

The properties of the aminolaevulinate synthetase of *M. denitrificans* are similar to those reported for the aminolaevulinate synthetases of a number of other systems which have been taken as evidence that this enzyme controls the rate of biosynthesis of haem from succinyl-CoA and glycine (for review see Burnham, 1969). The rate of synthesis of aminolaevulinate, catalysed by aminolaevulinate synthetase, is lower than the rate of its conversion into porphobilinogen even in extracts of cells grown anaerobically in defined nitrate medium where aminolaevulinate synthetase activity is highest. The activity of aminolaevulinate synthetase is markedly different in cells grown under different conditions, whereas the activities of aminolaevulinate dehydratase and of succinic thiokinase are about the same under all conditions of growth. Aerobically grown *M. denitrificans* has a lower cytochrome content (Porra & Lascelles, 1965) and a lower aminolaevulinate synthetase activity than cells grown anaerobically. Aminolaevulinate synthetase activity is markedly inhibited by low concentrations of haemin. The activity of the enzyme changes rapidly when additions are made to the growth medium. All these findings strongly suggest that aminolaevulinate synthetase is the rate-controlling enzyme in haem biosynthesis in *M. denitrificans*.

Aminolaevulinate synthetase activity is low in iron-deficient cells and it increases to the normal value in about 2 h on adding iron to a culture under conditions where protein synthesis can occur; Fe²⁺ and Fe³⁺ have no effect on enzyme activity *in vitro*. Thus it seems that in *M. denitrificans* iron controls aminolaevulinate synthetase in two different ways. It acts directly, in some unknown way, in the formation of the enzyme, and it also acts in a less-direct way as iron protoporphyrin to inhibit the activity of the enzyme. In other bacteria iron does not seem to be involved in the formation of aminolaevulinate synthetase. In *S. itersonii* (Ho & Lascelles, 1971) and in *R. sphaeroides* (Burnham & Lascelles, 1963) the

activity of the enzyme is the same in iron-deficient cells as in normal cells. These, and a number of other bacteria (Lascelles, 1962), respond to iron deficiency by excreting coproporphyrin. To explain this it has been suggested (cf. Lascelles, 1968) that in iron deficiency haem is synthesized at a decreased rate, the inhibition of aminolaevulinate synthetase activity is relieved, and thus aminolaevulinate and other intermediates in haem biosynthesis are formed at an increased rate. By contrast *M. denitrificans* excretes less coproporphyrin when grown in an iron-deficient medium. Stein *et al.* (1970) showed that ferric citrate had a marked synergistic effect on the induction of hepatic aminolaevulinate synthetase by allylisopropylacetamide. Their results suggested that iron stimulated the rate of synthesis of the enzyme rather than having an effect on its stability.

Effects of Ca²⁺ and Cu²⁺ on growth and aminolaevulinate synthetase activity

Initially, the results of growth experiments in non-AnalaR medium seemed to indicate that the concentration of Ca²⁺ was the only factor determining the extent of growth and the activity of aminolaevulinate synthetase. However, use of AnalaR medium showed that Cu²⁺ was also important since, in the absence of Cu²⁺, Ca²⁺ had little effect on growth or on aminolaevulinate synthetase activity. It seems that Ca²⁺ is required for the cells to take up Cu²⁺, or alternatively that Cu²⁺ is required for the cells to take up Ca²⁺. Comparison of the extent of growth obtained with low and high concentrations of Ca²⁺ in non-AnalaR medium and in AnalaR medium plus Cu²⁺ (Tables 3 and 5), suggests that the non-AnalaR medium used in these experiments contained about 0.1 µmol of Cu²⁺/litre. Batches of non-AnalaR medium used in some other experiments contained less Cu²⁺ than this because, as with AnalaR medium, even high concentrations of Ca²⁺ gave low growth and high activities of aminolaevulinate synthetase. Sodium succinate and K₂HPO₄ were the two non-AnalaR reagents that contained Cu²⁺, and it is probable that the different amounts of Cu²⁺ in different batches of these reagents were responsible for the non-reproducible results obtained in the initial experiments. The roles of Cu²⁺ and of Ca²⁺ in the growth of this organism are not known, nor is it known what compound or compounds are responsible for the yellow colour of the medium after growth in the presence of a high concentration of Cu²⁺. However, Cu²⁺ is involved in nitrite and hyponitrite reduction in *Neurospora crassa* (Medina & Nicholas, 1957), and it may play a similar role in *M. denitrificans*. If Cu²⁺ causes the low activities of aminolaevulinate synthetase, there are at least two ways in which it could act. Cu²⁺ may inhibit the

formation of the enzyme by interfering with the role of iron or, alternatively, it may bind directly to the enzyme and inactivate it. Whether or not such an inactivation occurs *in vivo* will depend on the intracellular concentration of Cu²⁺ and on the relative affinities for Cu²⁺ of aminolaevulinate synthetase and other cellular proteins.

These results may also be relevant to the inability to detect aminolaevulinate synthetase activity in other non-photosynthetic bacteria (cf. Lascelles, 1964; Burnham, 1969). These organisms may have been grown in the presence of high concentrations of Ca²⁺ and Cu²⁺, and tested for activity at the wrong stage of growth.

Loss of aminolaevulinate synthetase activity in vivo

Aminolaevulinate synthetase activity is stable when cultures of *M. denitrificans* grown anaerobically in media with low concentrations of Ca²⁺ and Cu²⁺ go into the stationary phase, and also when cells are resuspended in phosphate buffer. There is a rapid loss of activity when inhibitors of protein synthesis are added to cells suspended in medium, or when nitrate or air is added to cells suspended in buffer. The loss of activity is prevented by N₃⁻ and CN⁻. These results suggest that for loss of activity to occur the electron-transport system, or at least part of it, must be functioning and protein synthesis must be stopped. By analogy with the findings for this enzyme and other enzymes in other systems, there are two possible explanations for the loss of activity. The enzyme may be inactivated but not degraded, in which case reactivation *in vitro* should be possible under suitable conditions, or alternatively, the enzyme protein may be degraded. Inactivation without degradation has been shown for a number of enzymes. The activity of aminolaevulinate synthetase in cell-free extracts of *R. sphaeroides* can be changed markedly by treating the extracts in various ways (Marriott *et al.*, 1969, 1970; Warnick & Burnham, 1971; Tuboi & Hayasaka, 1972). The threonine dehydratase activity of *Bacillus licheniformis* is lost under conditions of glucose deficiency (Leitzmann & Bernlohr, 1968), but the quantity of enzyme protein estimated by immunoassay remains unchanged. Ornithine transcarbamoylase of *Saccharomyces cerevisiae* is inactivated by adding arginine to the culture, but activity can be restored by heating extracts (Bechet & Wiame, 1965). Nitrate reductase of *Chlamydomonas reinhardtii* is inactivated by adding NH₄⁺ to the culture, but activity can be restored by treating cell-free extracts with ferricyanide (Herrera *et al.*, 1972). Conditions for increasing the activity of aminolaevulinate synthetase in cell-free extracts have not yet been found.

Direct evidence for degradation of an enzyme can only be obtained by using methods which measure

the quantity of enzyme protein rather than its catalytic activity, although presumptive evidence can sometimes be obtained by using less rigorous techniques (see Schimke & Doyle, 1970). John *et al.* (1970) showed that the protein with isocitrate lyase activity in *Chlorella pyrenoidosa* disappeared at a rate of 9%/h when acetate-adapted cells were suspended in N₂-free medium and supplied with glucose. They also found that during growth the rate of turnover of this enzyme was slower than that of other cellular proteins and too slow to account for its disappearance in N₂-free medium, suggesting that a special degradative mechanism for this enzyme came into operation under these conditions. Nitrate reductase of *Ustilago maydis* (Lewis & Fincham, 1970) is inactivated *in vivo* by adding NH₄⁺ to the culture and the evidence suggests that the protein is degraded. The aminolaevulinate synthetase of liver mitochondria has a half-life of about 70 min (Marver *et al.*, 1966). Thus it appears that the changes in activity of nitrate reductase and of aminolaevulinate synthetase in different systems are brought about by different mechanisms. A number of processes involved in protein degradation have been described (cf. Nath & Koch, 1970, 1971; Prouty & Goldberg, 1972; Goldberg, 1971b; Katsunuma *et al.*, 1972). Some of these appear to operate under all conditions of growth and some only under special conditions such as nutritional starvation; some appear to act on special types of proteins and some are less specific.

The results obtained so far are not sufficient to decide which mechanism is responsible for the loss of aminolaevulinate synthetase activity in *M. denitrificans*, but it may be suggested tentatively that the enzyme does not turnover during growth, but that it is degraded rapidly under special conditions, for example when growth is changed from anaerobic to aerobic conditions, and when cultures that can still perform electron transport lose their ability to form protein.

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