Tetrapyrrole biosynthesis: N-methyl-N'-nitrosoguanidine-induced mutants of *Propionibacterium shermanii*

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SUMMARY

An isolation method for N-methyl-N'-nitrosoguanidine-induced catalase negative mutants of *P. shermanii* based on replica plating is described. In contrast to previous methods, it extends to the early stages of tetrapyrrole biosynthesis which are common in both corrins and porphyrins. It may thus aid in elucidating the mechanism and control of porphyrin and corrin biosynthesis. Some preliminary results are discussed.

1. INTRODUCTION

P. shermanii mutants that are unable to synthesize dA-B_{12}* (the form of B_{12} that is synthesized *in vivo*) have been induced by a variety of mutagens including dimethyl sulphate (Mashur, Vorob'era & Iordan, 1971; Vorob'eva *et al.* 1971), MNNG (Vorob'eva, Baranova & Thanh, 1973) nitrosomethyl urea (Arkad'eva & Kalenik, 1971) and ethyl methanesulphonate (Pedziwilk, 1971). All these studies have focused on comparing the fermentations and fermentation products of mutant and parent strain (Fig. 1); in no instance was an attempt made to locate a biochemical defect.

Prompted by the renewed interest in the biosynthesis and regulation of porphyrins and corrins (Fig. 2; Bykhovskii, Zaitseva & Bukin, 1969; Sato, Shimizu & Fukui, 1971; Scott, 1975; Friedman, 1975) we have developed a selection method for *P. shermanii* mutants based on the haem pathway (which is simpler and better understood than the B_{12} pathway). This method may provide an approach to (*a*) unequivocally determine the branching point of corrin biosynthesis at the enzyme level; (*b*) investigate the regulation of tetrapyrrole biosynthesis; (*c*) produce strains that can synthesize corrins only, thus eliminating the large porphyrin background that we and other investigators have encountered in studying the formation of the corrin nucleus.

* *Abbreviations*: ALA, δ-amino levulinic acid; dA-B_{12}, 5'-deoxyadenosyl-B_{12}; cobester, cobyrinic acid heptamethyl ester; copro, coproporphyrin; copro'gen, coproporphyrinogen; DTT, dithiothreitol; GSH, glutathione; MNNG, N-methyl-N'-nitro-N-nitroso guanidine; PBG, porphobilinogen; proto, protoporphyrin; SAM, S-adenosylmethionine; uro, uroporphyrin; uro'gen, uroporphyrinogen.

2. MATERIALS AND METHODS

(i) *Materials*

ALA, SAM, DTT, Tween 80, L-methionine, copro-III-tetra-methyl ester, proto-IX-dimethyl ester, MNNG and haemin were obtained from Sigma; CN-B_{12} (crystalline) from Merck; tris base from Mann; yeast extract, casein hydrolysate (acid hydrolysed) and casein hydrolysate (enzyme hydrolysed) from Difco, ^{14}C-PBG was a generous gift of Professor Shemin. Uro III was prepared by Dr G. H. Temme, according to published procedures (Tarlton, MacDonald & Baltazzi, 1960; Franck, Gantz & Hüper, 1972). Uro'gen III was prepared by sodium amalgam reduction of uro III (Falk, 1964) under oxygen-free nitrogen (obtained by passing nitrogen through an alkaline solution of pyrogallol) in the dark and was used immediately.

Propionibacterium shermanii (ATCC 9614) was purchased from the American Type Culture Collection. Both parent strain and subsequent mutant strains were stored at -10 °C after adding 4 ml of 80 % glycerol to 10 ml of log phase (24 h)* cultures. When needed, the frozen cultures were quickly thawed and used to inoculate (5 % inocula) liquid cultures.
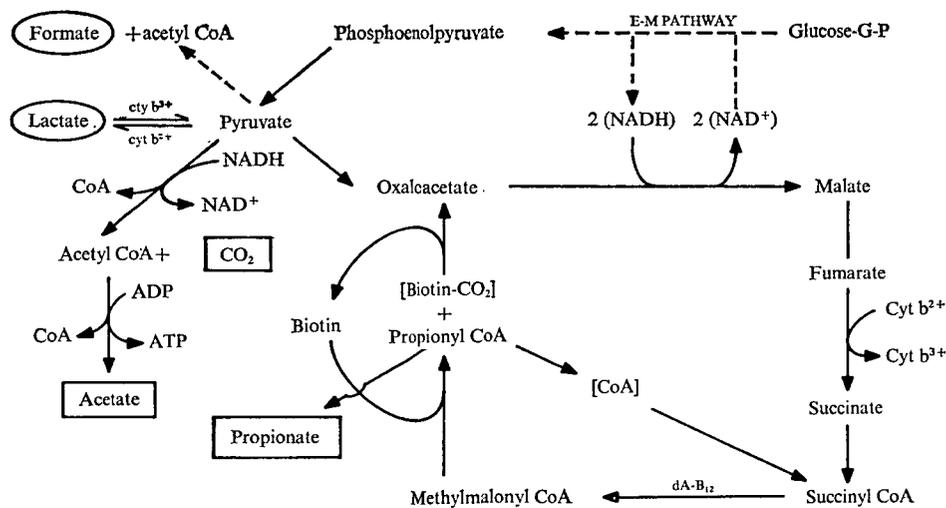

Fig. 1. Diagram of propionic acid fermentation in *P. shermanii*, derived from Vorob'eva *et al.* (1971) and de Vries *et al.* (1972). Products that normally accumulate are enclosed in boxes; those that accumulate during B_{12} deficiency are enclosed in circles. Brackets indicate transient intermediates; broken arrows indicate multistep reaction.

(ii) *Methods*

Media and culturing conditions. Regular medium contained (per litre deionized water): casein hydrolysate (acid hydrolysed), 12·5 g; casein hydrolysate (enzymic), 12·5 g; yeast extract, 5·0 g; glucose (autoclaved separately and then added aseptically) 6·0 g; KH_2PO_4, 1·76 g; K_3PO_4, 1·76 g; $MgCl_2.6H_2O$, 0·4 g; $FeSO_4.7H_2O$, 0·010 g. BH medium contained in addition B_{12} and haemin, in 2 μg/ml final concentration each. Since haemin rapidly oxidizes to hematin, cells were actually growing in the presence of the latter. Regular agar was prepared by mixing melted 3 % agar with equal volume of double-

* Growth is exponential up to 60 h (N.H.G. and J.P. unpublished observations).
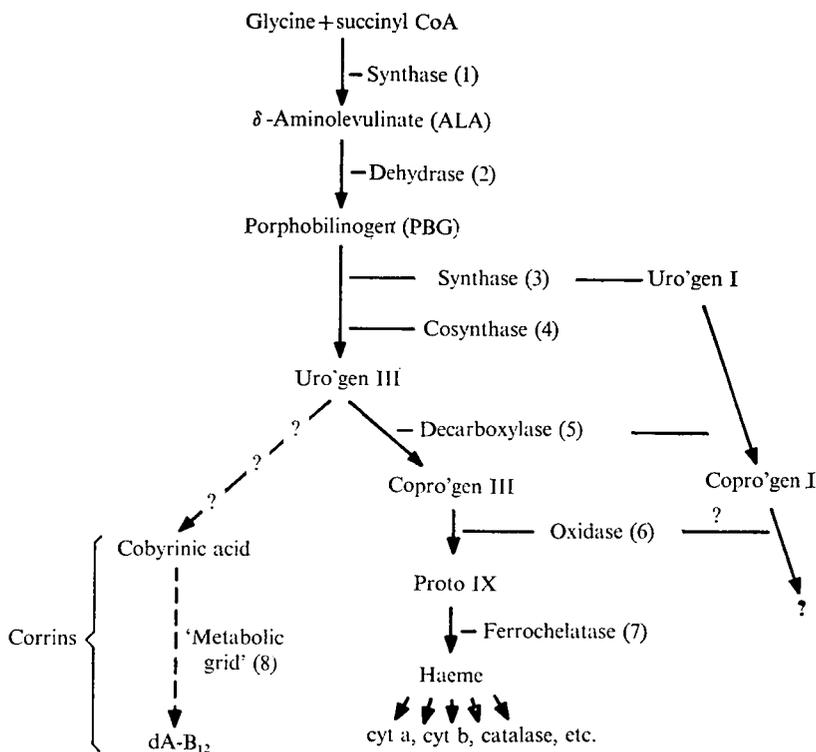
Fig. 2. The pathway to porphyrins and corrins. Numbers in parentheses next to individual enzymes relate to references at the end of the text as follows: (1) Menon & Shemin, 1967; (2) Walerych, 1963; (3) Bogorad, 1958a; (4) Bogorad, 1958b; (5) Hoare & Heath, 1959; (6) Granick & Mauzerall, 1958; (7) Porra & Jones, 1963; (8) Wagner, 1966. No intermediates have been isolated between uro'gen III and cobyrinic acid. The steps beyond cobyrinic acid have been omitted for the sake of clarity.

strength regular medium. BH agar contained in addition B_{12} and hemin, in 2 μg/ml final concentration each. Both haemin and B_{12} were sterilized by filtration (Nalgene filter unit; 0·2 μm pore diameter) as 1 mg/ml aqueous solution and were then added to the sterile regular medium or molten agar. Because haemin is insoluble in water, haemin (10 mg) was first dissolved in 50 % aqueous ethanolamine (2 ml) and then water was added to make up to the desired volume (10 ml). Haem, B_{12} and BH media were handled in dim light. Incubations in BH media were carried out in the dark.

Liquid cultures were grown in two stages. (a) The organism was grown in a 50 ml screw-cap culture tube filled to the top with BH medium at 28–30 °C for 4 days. (b) The culture was transferred into a 500 ml Erlenmeyer flask containing 200 ml of BH medium and was grown at 20–22 °C anaerobically for 3 days. During this stage the pH was daily adjusted to 7·0 with 10 % aqueous Na_2CO_3 and, at the same time, 2·5 ml of 20 % sterile glucose solution was added. Cells were harvested by centrifugation and washed twice with 30 ml of 0·05 M phosphate buffer (pH 7·6). Yields varied among strains, ranging from a few mg up to 3 g of cells.

For plating, 0·1 ml of diluted culture was pipetted on an agar plate and spread with a sterile glass spreader. Dilutions were carried out using 0·05 M phosphate buffer (pH 7·6) containing 0·05 % Tween 80. Plates were incubated at 30 °C in anaerobic jars (Gaspak Anaerobic System).

Fresh solutions of MNNG in sterile 0·05 M Tris-maleate buffer (pH 6·0; Adelberg, Mandell & Ching Chen, 1965) were made each time and were used immediately (sterility was checked by plating a 0·1 ml aliquot).

(iii) *Haemin and B_{12} uptake*

Freshly harvested cells (wet weight: 20 g) were suspended in 80 ml of 0·05 M phosphate buffer by briefly (15 sec) homogenizing in a Sorvall omnimixer. They were then equally divided into 20 portions, each of which was added to 45 ml of the above buffer in a 125 ml Erlenmeyer flask. To the bacterial suspensions haemin (or B_{12}) was added to a final concentration of 20 μg/ml and the samples were maintained at 30 °C with a parallel control at 0 °C. After early experiments, it was found that at this concentration haemin caused a 90 % decrease in OD after 24 h relative to the control (no haemin); thus, in all other experiments a 2 μg/ml final concentration was used which caused a 20 % decrease. B_{12} was used in the same concentration as haemin, although in its case neither uptake nor growth inhibition could be detected.

After various intervals haemin (or B_{12}) was determined spectrophotometrically in the supernatant obtained after centrifugation in the cold and in one washing with 30 ml of 0·05 M phosphate buffer. Haemin measurements were made at 380 nm (Sasarman *et al.* 1968); B_{12} measurements at 365 nm after the addition of KCN to 0·01 % final concentration (Wagner & Berhauer, 1964).

(iv) *Mutagenesis*

Four 20 ml log-phase (24 h) cultures of *P. shermanii* were harvested by centrifugation and washed with 5 ml of 0·05 M phosphate buffer (pH 7·6). Each culture was then suspended in 10 ml of MNNG solution of the appropriate concentration (Table 1) and was incubated at 30 °C for 30 min. It was subsequently centrifuged, washed twice with 5 ml of the above buffer and finally resuspended in 50 ml of BH medium. After 24 h incubation (to allow segregation of pure colonies), 1 ml aliquots of the cultures were removed, serially diluted and plated on agar. Plates were incubated at 28–30 °C for 6 days. From the number of resulting colonies survival was determined. The colonies (1 mm diameter were transferred ('gridded') on to BH agar plates (100 colonies per plate) by the method of Low (1973). After 4 days incubation, the master plates were replica plated (Lederberg & Lederberg, 1952) on to regular plates and were grown for another 4 days. The percentage of mutants was determined by the catalase test described below.

(v) *Selection of mutants*

(a) *Catalase activity*. This was determined qualitatively by adding one drop of 3 % hydrogen peroxide solution on to each colony on the replica (regular agar) plates. The plate was held tilted so that the reagent would not flood it. Any catalase negative (Cat$^-$) colonies, identified by the absence of small bubbles on their surface, were subcultured from the corresponding colonies on the original (BH) plate and purified by dilution plating on BH agar and isolation of single colonies. Twenty of these colonies were gridded on regular agar and tested again for catalase activity.

(b) *Test for porphyrins and corrins*. The cells harvested from 250 ml liquid culture (3 + 4 days) were resuspended in 10 ml of 0·2 M phosphate buffer in a 50 ml Erlenmeyer flask. Next, 0·2 ml of 0·1 M-CoCl$_2$ and 0·5 ml aqueous solution containing 10 mg GSH, 5 mg DTT, 3 mg methionine and 1 mg ALA were added to the cell suspension. After briefly flushing with pure nitrogen the flask was stoppered tightly, wrapped in aluminium foil and incubated in a Dubnoff incubator (100 oscillations/min) at 30 °C for 8 h. In all sets of incubations the parent strain was included as a control and produced approx. 0·2 μm each of porphyrins and corrins.

The incubation mixture, after addition of 0·5 ml of 1 M-KCN, was lyophilized to dryness and then treated with 10 ml of 10 % H_2SO_4/MeOH (r.t.; 24 hr). After neutralization with saturated $NaHCO_3$, corrin and porphyrin methyl esters were extracted with 2×10 ml CCl_4; the course of extraction being followed by the disappearance of red fluorescence (under UV) from the aqueous layer. They were then separated by chromatography on silica gel plates (Brinkman, Silplate-22) developed with benzene–ethyl acetate–methanol (70:24:6). KCN was added (0·5 mg/ml) to all solvents used in the work-up. Porphyrins were detected by exposure to 350 nm UV light; corrins (cobester) were visible in room light as a purple band. R_F values obtained were: proto dimethyl ester, 0·9; copro tetra-methyl ester, 0·5; uro octamethyl ester, 0·6; cobester, 0·1.

(c) *Enzymatic analyses.* To 3 g of freshly harvested cells 6 ml of 0·2 M phosphate buffer (pH 7·6) were added and the mixture was sonicated in a Branson sonicator (10–20 kc; 125 W output) for 8 min with cooling. The sonicate was centrifuged at 30000 g for 10 min and the supernatant was used for the incubations which were done as in (ii) except that PBG or uro'gen III was used instead of ALA and SAM instead of methionine. At physiological pH cells are impermeable to uro'gen or PBG (N.H.G. unpublished observations). Therefore intact cells cannot be used in incubations with these substrates.

3. RESULTS AND DISCUSSION

We were interested in the biosynthesis of tetrapyrroles and knew that catalase, a readily assayed haemoprotein, is normally present in *P. shermanii*. Therefore, as a preliminary screen for mutants which are genetically blocked in tetrapyrrole biosynthesis, we decided to search for Cat⁻ mutants. One of the problems we anticipated was the possibility that the haemin-deficient subgroup of Cat⁻ mutants would not grow at all unless haemin was supplied exogenously. Cytochromes *a* and *b* – which, like catalase have haem as a prosthetic group – are normally present in *P. shermanii* and are essential for the growth of this organism (de Vries, Van Wijck-Kapteijn & Stouthamer, 1972). Thus, to determine whether haemin-deficient mutants could be rescued by addition of haemin to the medium, the parent strain was tested for haemin uptake. As shown in Fig. 3, haemin is taken up by the cells. The uptake increases with time and shows a slight temperature dependence. We also observed (Fig. 3) that B_{12} was not appreciably taken up. This is in contrast to the results of Bykhovskii *et al.* (1969), who worked with a different strain of *P. shermanii* and observed B_{12} uptake. The vitamin, however, is not crucial for cell survival; methionine (whose formation dA·B_{12} mediates) is supplied in casein, and propionic acid formation (Fig. 1) is a feature of the stationary (rather than growth) phase (Mashur *et al.* 1971).

With the problem of rescuing the mutants thus provisionally resolved, we proceeded with the mutagenesis. As shown in Table 1, the survival rate of *P. shermanii* decreased drastically with increasing concentrations of MNNG. Concomitantly, the number of Cat⁻ mutants per survivor showed a moderate increase. It was also observed that cold storage of mutagenized cells beyond two weeks resulted in very poor recovery of Cat⁻ mutants. This suggests that many potential Cat⁻ mutants may be lost in the 24 h post MNNG treatment incubation. Catalase, as it is well known, is needed for detoxifying hydrogen peroxide, which most organisms produce in the presence of oxygen. Our conditions, up to the post-mutagenesis dilution plating, were not anaerobic.

After screening 7000 colonies derived from MNNG-treated cultures, 43 Cat⁻ mutants were obtained. Characteristics of the parent and some mutant strains are outlined in Table 2. All strains grew very slowly and, in the case of growth on agar plates, stringent anaerobic conditions were applied (de Vries *et al.* 1972). Colonies of the parent strain were cream coloured, smooth, convex and shiny. Most of the mutants had similar colony
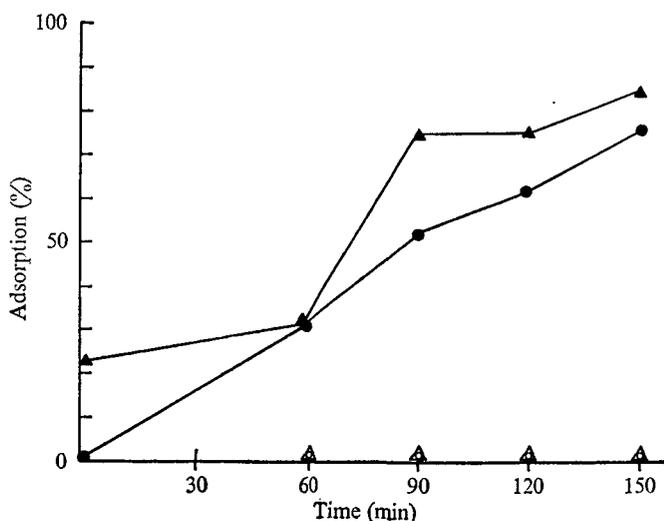
Fig. 3. Haemin and B_{12} uptake by *P. shermanii* ATCC 9614 (parent strain). (▲) Haemin (30 °C); (●) Haemin (0 °C); (△) B_{12} (30 °C); (○) B_{12} (0 °C). OD readings of supernatant and washings were combined.

Table 1. *Effect of various concentrations of MNNG on mutation rate and cell death*

MNNG (mg/ml for 30 min)	Survival rate (%)	Colonies analysed, total no.	Cat⁻ mutants	
			Total no.	%
0	100	2000	.	< 0·05
0·2	1	1000	1	0·1
0·4	0·1	3000	13	0·5
0·8	0·01	3000	29	1

Table 2. *Comparison of some mutants to the parent strain*

(Porphyrinogens are autooxodized to porphyrins; corrins are predominantly cobyrinic acid; $\pm$, low level.)

Strain	Gram stain	Colony morphology	Porphyrins formed			Corrins formed	Growth in liquid culture
			Uro	Copro	Proto		
Parent	+	Cream coloured	+	+	+	+	+ + + +
Cat 9	+	White dwarf	−	−	−	−	+
Cat 10	+	Cream coloured	±	±	±	±	+ + +
Cat 13	+	White dwarf	−	−	−	−	+ + +
Cat 41	+	Cream coloured	±	±	±	−	+

morphology. Notable exceptions were Cat 9 and Cat 13, which formed small white colonies. This feature, stable after subculturing, is similar to that observed by Pedziwilk (1971) in *P. shermanii* mutants that were both B_{12} deficient and catalase negative. Biochemical tests (fermentation of sugars, milk coagulation, indole formation, nitrate reduction) indicated the presence of additional mutations in a few of the mutants. The

possibility that these mutants were contaminants was ruled out on the grounds of colony morphology, gram stain, cobyrinic acid production and the characteristic 'swiss-cheese' odour.

Of the 43 mutants examined, four were found to have the biosynthetic block between ALA and proto IX (Fig. 2, Table 2). The rest were considered to be defective either before ALA or after proto IX. One of the porphyrin-forming mutants (Cat 41) failed to form corrinoids. Although the trivial explanation of multiple mutations cannot be excluded, two other possible explanations for the phenotype of Cat 41 may be considered: (*a*) the cobalt-inserting enzyme and ferrochelatase share a common factor which is defective (the proto IX → haem step could not be unequivocally determined because of possible loss of the haem iron during post-incubation work-up); (*b*) the only uro'gen formed is the I isomer (i.e. the block lies in the uro'gen III cosynthase; Fig. 2). In incubations with either the parent or mutant strains, accumulation of copro'gen occurred which is consistent with the requirement for molecular oxygen of the copro'gen III to proto IX conversion.

The multiple defects (ALA → PBG; PBG → uro'gen III; uro'gen III → copro'gen III) observed with Cat 13 (and to a slightly lesser degree Cat 9) might be the result of a regulatory mutation. Alternatively, MNNG might introduce multiple mutations as in *E. coli* (Guerola, Ingraham & Cerda-Olmedo, 1971; Tien & White, 1968). The fact that we did not detect single genetic blocks between ALA and uro'gen III might possibly indicate enzyme multiplicity in the common part of the pathway (Fig. 2). Although there is a precedent in *R. spheroides* where two ALA synthases have been found (Tuboi, Kim & Kikuchi, 1970) we have not yet screened a sufficiently large population to be able to suggest a similar situation.

In conclusion, *P. shermanii*, because it is well endowed with enzymes that make both porphyrins and corrins, is an attractive system for studying the biosynthesis of these compounds and its regulation. The outlined methodology permits the beginning of a genetic study of tetrapyrrole synthesis in this organism.

REFERENCES

ADELBERG, E. A., MANDEL, M. & CHING CHEN, G. C. (1965). Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoquanidine in *Escherichia coli* K-12. *Biochemical and Biophysical Research Communications* **18**, 788–795.

ARKAD'EVA, Z. A. & KALENIK, N. M. (1971). Physiological and biochemical characteristics of some mutants of *Propionibacterium shermanii*. *Microbiologiya* **40**, 880–885.

BOGORAD, L. (1958*a*). The enzymatic synthesis of porphyrins from porphobilinogen. I. Uroporphyrin I. *Journal of Biological Chemistry* **233**, 501–509.

BOGORAD, L. (1958*b*). The enzymatic synthesis of porphyrins from porphobilinogen. II. Uroporphyrin III. *Journal of Biological Chemistry* **233**, 510–515.

BYKHOVSKII, V. YA., ZAITSEVA, N. I. & BUKIN, U. N. (1969). Some aspects of the regulation of vitamin B_{12} and porphyrin biosynthesis in *Propionibacterium shermanii*. *Doklady Akademii Nauk. SSSR* **185**, 459–461.

DE VRIES, W., VAN WIJCK-KAPTEIJN, W. M. C. & STOUTHAMER, A. H. (1972). Influence of oxygen on growth, cytochrome synthesis and fermentation pattern in propionic acid bacteria. *Journal of General Microbiology* **71**, 515–524.

FALK, J. E. (1964). *Porphyrins and Metalloporphyrins*, New York: Elsevier.

FRANK, B., GANTZ, D. & HÜPER, F. (1972). Total synthesis of $[\alpha,\gamma$-$^{14}C_2]$ uroporphyrin III. *Angewandte Chemie* International Edition in English, **11**, 420.

FRIEDMAN, H. C. (1975). Biosynthesis of corrinoids. In *Cobalamin: Biochemistry and Pathology* (ed. B. M. Babior), pp. 77–89. New York: Wiley.

Granick, S. & Mauzerall, D. (1958). Enzymic formation of protoporphyrin from copro-porphyrinogen III. *Federation Proceedings* **17**, 233.

Guerola, N., Ingraham, J. L. & Cerda-Olmedo, E. (1971). Induction of closely linked multiple mutations by nitrosoquanidine. *Nature New Biology* **230**, 122–123.

Hoare, D. S. & Heath, H. (1959). The biosynthesis of porphyrins from porphobilinogen by *Rhodopseudomanas spheroides*. *Biochemical Journal* **73**, 679–690.

Lederberg, J. & Lederberg, E. M. (1952). Replica plating and indirect selection of bacterial mutants. *Journal of Bacteriology* **63**, 399–406.

Low, B. (1973). Rapid mapping of conditional and auxotrophic mutations in *Escherichia coli* K-12. *Journal of Bacteriology* **113**, 798–812.

Mashur, V. A., Vorob'eva, L. I. & Iordan, E. P. (1971). Fermentation caused by a mutant of propionic acid bacteria that do not form B_{12} coenzymes. *Prikladnaya Biokhimiya Mikrobiologiya* **7**, 552–555.

Menon, A. I. & Shemin, D. (1967). Concomitant decrease of enzymic activities concerned with the synthesis of coenzyme B_{12} and of propionic acid in *Propionibacteria*. *Archives of Biochemistry of Biophysics* **121**, 304–310.

Pedziwilk, F. (1971). Ethyl methanesulfonate (EMS-induced mutants of *Propionibacterium Shermanii*. *Acta Microbiologica Polinica* B **3**, 79–89.

Porra, R. J. & Jones, O. T. G. (1963). Studies in ferrochelatase. 1. Assay and properties of ferrochelotase from a pig-liver mitochondrial extract. *Biochemical Journal* **87**, 181–185.

Sasarman, A., Surdeanu, M., Szegli, G., Horodniceanu, T., Greceanu, V. & Dumitrescu, A. (1968). Hemin-deficient mutants of *Escherichia coli* K-12. *Journal of Bacteriology* **96**, 570–572.

Sato, K., Shimizu, S. & Fukui, S. (1971). Studies on the corinoids and porphyrins in Streptomycetes. V. Effect of additions on porphyrin excretion and δ-aminolevulinic acid dehydratase in *Streptomyces olivaceus*. *Agr. Biol. Chem.* **35**, 344–350.

Scott, A. I. (1975). Concerning the biosynthesis of vitamin B_{12}. *Annals of the New York Academy of Sciences* **244**, 356–370.

Tarlton, E. J., MacDonald, S. F. & Baltazzi, E. (1960). Uroporphyrin 3. *Journal of the American Chemical Society* **82**, 4389.

Tien, W. & White, D. C. (1968). Linear sequential arrangement of genes for the biosynthetic pathway of protoheme in *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences, U.S.A.* **61**, 1392–1398.

Tuboi, S., Kim, H. J. & Kikuchi, G. (1970). Occurrence and properties of two types of δ-aminolevulinate synthetase in *Rhodopscudomonas spheroides*. *Archives of Biochemistry & Biophysics* **138**, 147–159.

Vorob'eva, L. I., Baranova, N. A. & Thanh, C. T. (1973). Mutagenic action of N-methyl-N-nitrosoguanidine on propionic acid bacteria. *Mikrobiologiya* **42**, 301–306.

Vorob'eva, L. I., Mashur, V. A., Baranova, N. A., Kopelevich, V. M. & Evdokimova, G. S. (1971). Enzymes of propionic acid fermentation in mutant and parent forms of *Propionibacterium shermanii*, *Mikrobiologiya* **40**, 686–690.

Wagner, F. (1966). Vitamin B_{12} and related compounds. *Annual Review of Biochemistry* **35**, 405–434.

Wagner, F. & Bernhauer, K. (1964). New aspects of the structure of corinoid coenzymes. *Annals of the New York Academy of Sciences* **112**, 580–589.

Walerych, W. (1963). δ-Aminolevulinic acid dehydratese from propionic acid bacteria. *Acta Biochimica Polonica* **10**, 243–252.