

Coproporphyrinogenase in Tobacco (*Nicotiana tabacum* L.)

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1. Coproporphyrinogenase was extracted and purified from tobacco (*Nicotiana tabacum* L.). Enzyme activity was mainly located in mitochondria rather than in chloroplasts. The enzyme was purified by differential centrifugation, ammonium sulphate fractionation, calcium phosphate gel adsorption and dialysis. A 69-fold final purification was obtained. 2. An apparent K_m value of $3.6 \times 10^{-5} M$ was found, the value being largely dependent on the amount of coproporphyrin III recovered after reduction with sodium amalgam to coproporphyrinogen III. Protoporphyrin formation was linear up to 3 h and decreased with further incubation. The enzyme activity increased with the concentration of enzyme protein up to $30 \mu g/ml$ of solution. 3. Enzyme activity was greatly enhanced by increasing Fe^{2+} concentrations up to 0.5 mM, beyond which inhibition occurred. Co^{2+} and Mn^{2+} were also found to activate at low concentrations (0.1 mM) and inhibit at higher concentrations (5 mM). Fe^{3+} and Cu^{2+} , both at 0.1 mM, and *o*-phenanthroline and EDTA, each at 1 mM, were found to be inhibitory.

The requirement for iron for the biosynthesis of chlorophyll both in higher plants and in photosynthetic bacteria has been known for decades (Gris, 1844; Van Niel, 1944). Iron is not a component of any chlorophyll precursor. It has been suggested that iron may be required in enzymic reactions in the overall pathway leading to chlorophyll biosynthesis (Lascelles, 1962). The suggested reactions are the condensation of succinyl-CoA and glycine into δ -aminolaevulate, the conversion of coproporphyrinogen III into protoporphyrin IX and the turnover of magnesium protoporphyrin monomethyl ester.

Pappenheimer (1947) showed that *Corynebacterium diphtheriae* accumulated porphyrins together with toxin when grown in iron-deficient media. The yield of porphyrin decreased when iron was increased beyond a critical concentration. An accumulation of porphyrin, predominantly coproporphyrin III, has since been found in many non-photosynthetic organisms and photosynthetic bacteria grown under conditions of low iron concentration (Van Niel, 1944; Lascelles, 1956, 1962). In photosynthetic organisms increasing the concentrations of iron suppressed the excretion of porphyrin but increased the formation of bacteriochlorophyll (Lascelles, 1956, 1962).

The addition of iron did not suppress porphyrin

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synthesis when δ -aminolaevulate was supplied as substrate for *Rhodospseudomonas spheroides*. Coproporphyrin III was the predominant product formed. Considerable amounts of protoporphyrin IX were also found, in contrast with experiments in which iron-deficient conditions were used (Lascelles, 1962). The influence of iron on the concentration of coproporphyrin III formed from δ -aminolaevulate suggests that iron may participate in the coproporphyrinogenase-catalysed conversion of the propionic side chains of coproporphyrin III into the vinyl side chains of protoporphyrin IX.

It is noteworthy that mitochondrial coproporphyrinogenase is inhibited by *o*-phenanthroline and 8-hydroxyquinoline 5-sulphate, chelating agents with a high affinity for iron (Sano & Granick, 1961; Granick & Mauzerall, 1958).

In contrast with the above evidence suggesting a role of iron in the synthesis of protoporphyrin IX, Marsh, Evans & Matrone (1963*a,b*) reported that δ -aminolaevulate was converted into both protoporphyrin IX and chlorophyll by iron-deficient leaf discs (from cow-pea) at the same rate as by normal leaf discs. Batlle, Benson & Rimington (1965) were not able to demonstrate the presence of any essential metal in coproporphyrinogenase purified from rat liver mitochondria. Carell & Price (1965) observed an accumulation of coproporphyrin III and a decreased rate of protoporphyrin IX formation in *Euglena gracilis* only with extreme iron deficiency where growth was limited.

The work of Hsu & Miller (1965) suggested that

the conversion of coproporphyrinogen III into protoporphyrin IX was an iron-requiring step in the biosynthesis of chlorophyll in higher plants. It was found that δ -amino [^{14}C]laevulate was predominantly incorporated into [^{14}C]coproporphyrin III in leaf discs from normal and chlorotic plants. The concentration of [^{14}C]coproporphyrin III was markedly decreased when iron was added to the incubation media. Incorporation of δ -amino[^{14}C]laevulate into chlorophyll was greater in normal leaf discs, but addition of iron to chlorotic leaf discs gave an incorporation rate almost equal to that of the normal discs.

The present study was initiated to purify and characterize the enzyme coproporphyrinogenase from tobacco and to determine its requirement for metals.

MATERIALS AND METHODS

Plants. *Nicotiana tabacum* L. (var. Havana 38) was grown in Hoagland's nutrient culture medium at an iron concentration of 2.5 p.p.m. (Chelate 138 Fe, sodium ferric ethylenediamine 3,5-dihydroxyphenylacetate; Geigy Chemical Corp., New York, N.Y., U.S.A.) under controlled environmental conditions.

Coproporphyrin III. Coproporphyrin III tetramethyl ester from human porphyria (Calbiochem, Los Angeles, Calif., U.S.A., and Sigma Chemical Co., St Louis, Mo., U.S.A.) was hydrolysed with 6 M-HCl for 1 day at 25°C in the dark and precipitated by adjusting the pH to 3.6 with 30% (w/v) NaOH. The precipitated porphyrin was separated by centrifugation, washed twice with 0.1 M-sodium acetate buffer, pH 3.6, and dissolved in 0.01 M-KOH to give a final concentration of 0.5 mM.

Coproporphyrinogen III. Coproporphyrinogen III was prepared as outlined by Batlle *et al.* (1965). Coproporphyrin III (0.5 mM) was reduced with freshly ground 3% sodium amalgam (1 g/ml of solution) under N_2 in dim red light. The porphyrin was reduced to the colourless porphyrinogen in a few minutes. The solution was filtered by suction through a sintered-glass funnel into a tube containing 2 M-potassium thioglycollate to give a final concentration of 0.05 M and titrated to pH 7.4 with 40% (w/v) H_3PO_4 . The resulting solution was diluted to a volume double that of the original solution with 0.01 M-tris-HCl buffer, pH 7.4, and used immediately. The yield of coproporphyrinogen III was determined after reoxidation of an acidic mixture of coproporphyrin III by exposure to light and air for 20–30 min at room temperature.

Sodium amalgam was prepared by the method of Sano & Granick (1961) and Read & Lucarini (1925). Sodium metal was melted in a Pyrex flask containing toluene on a hot-plate. After removal of heat, mercury (sufficient to give 3% sodium amalgam) was added dropwise with shaking. A solid amalgam was formed in seconds. It was cooled on filter paper, washed with light petroleum (30–60°C), air-dried, crushed in an iron mortar and stored in an N_2 -flushed test tube until used.

Enzyme preparation. Leaf material (100 g) from tobacco (Havana 38) was cut into small pieces and added to 180 ml of an extraction medium containing 0.5 M-sucrose, 0.05 M-

ascorbic acid and 0.01 M-tris-HCl buffer, pH 7.4. It was homogenized in the cold for six periods of 30 s each in a Waring blender. After filtration through four layers of cheesecloth by suction, the extract was centrifuged at 17000g for 30 min to precipitate chloroplasts (Gorham, 1955), mitochondria, grana and broken chloroplasts (Axelrod, 1955). In experiments comparing activity in different cellular fractions the extract was centrifuged at 1000g for 15 min (chloroplast fraction) and then at 17000g for 30 min (mitochondrial fraction). The precipitate was suspended in 40 ml of 0.01 M-tris-HCl buffer, pH 7.4, and ultrasonicated for 5 min in an ice bath. The cellular debris was removed by centrifugation for 10 min at 17000g.

$(\text{NH}_4)_2\text{SO}_4$ was added to the cooled extract to give 50% saturation. After 1 h the solution was centrifuged at 11750g for 10 min at 1°C. The supernatant was brought to 75% saturation with $(\text{NH}_4)_2\text{SO}_4$ and after 10 min the solution was centrifuged at 11750g for 10 min. Both precipitates contained low coproporphyrinogenase activity and were discarded. Calcium phosphate gel (40 mg dry wt. of gel/ml), prepared by the method of Colowick (1955), was added to the supernatant in the proportion 1:5 (v/v). After being stirred for 10 min the gel was removed by centrifugation at 1060g for 10 min.

The enzyme in the above supernatant was dialysed against 0.01 M-tris-HCl buffer, pH 7.4 (two changes), in a cold-room for 16 h. The solution obtained was the partially purified enzyme and was used for kinetic studies, including those on metal requirements.

Identification of protoporphyrin IX. The product of the coproporphyrinogenase-catalysed reaction, protoporphyrin IX, was extracted from the assay mixture and chromatographed by the methods of Falk (1961). Protoporphyrin IX was extracted into ether, vacuum-dried, dissolved in a minimum volume of 2 M-KOH and spotted on a t.l.c. plate coated with silica gel (0.25 mm thick). The plate was developed with 2,6-lutidine-water (5:3, v/v) in a chamber saturated with NH_3 vapour. Markers of coproporphyrin III and protoporphyrin IX were also dissolved in 2 M-KOH and applied to each plate. The R_F values of coproporphyrin III and protoporphyrin IX on the silica gel plate were 0.39 and 0.69 respectively. Protoporphyrin IX was detected under u.v. light. The chromatogram indicated the presence of protoporphyrin IX in the standard assay mixture. Only traces were found in the assay mixture containing boiled extract. The coproporphyrin III stock solution contained about 3 ng of protoporphyrin IX/ μg of coproporphyrin as contaminant.

Assay of coproporphyrinogenase activity. The enzyme preparation was assayed by the method of Batlle *et al.* (1965). The reaction mixture, which contained 1 ml of the enzyme extract (23–30 μg) and 1 ml of freshly prepared coproporphyrinogen III (made by reduction of 0.5 ml of 0.5 mM-coproporphyrin III stock solution), was incubated aerobically in the dark at 30°C for 2 h with mechanical shaking at about 90–100 oscillations/min. After incubation, 0.33 ml of conc. HCl was added to each mixture to stop the reaction. The mixture was then exposed to light and air for 20–30 min at room temperature to convert any porphyrinogens into porphyrins. The precipitated protein was removed by filtration through Whatman no. 1 filter paper, washed with 3×3 ml and then with 2×1.5 ml of 5% (v/v) HCl. The filtrate and washings were combined.

The acid solution was promptly adjusted to pH 3.2 with saturated sodium acetate and extracted into ether. Coproporphyrin was extracted from the ether by shaking with 3 × 6 ml of 0.36% HCl. Protoporphyrin was then extracted with 3 × 3 ml of 5% HCl and diluted to 10 ml with the same acid. The extinction of the solution was measured at 380, 405 and 430 nm and the porphyrin concentrations were calculated by using the correction formulas of Rimington & Sveinsson (1950). Boiled enzyme incubated with the same concentration of coproporphyrinogen III was used as control and any protoporphyrin IX found was subtracted from the amount determined under the experimental conditions.

Enzyme unit. One unit of coproporphyrinogenase was defined as the amount of enzyme that catalysed the formation of 1 μmol of protoporphyrin IX/min from coproporphyrinogen III under the standard conditions described above. Specific activity was defined as no. of units/mg of protein.

Determination of iron and protein contents. Protein content was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Casein standard was used (Sigma Chemical Co.). Iron content was determined as outlined by Cameron (1965). The enzyme preparation (0.5 ml) was added to 0.1 ml each of 70% (w/v) HClO₄ and 30% (w/v) H₂O₂ and digested for 30 min at 100°C. The mixture was cooled and the iron reduced to ferrous form with 0.1 ml of freshly prepared 10% (w/v) hydroxylamine hydrochloride solution. After 5 min 1 ml of *o*-phenanthroline (0.5% in 50% ethanol) was added, followed immediately by 1 ml of pyridine. The solution was diluted to 10 ml with water and the extinction determined at 509 nm. The concentration of Fe²⁺ was obtained from a standard curve constructed by use of standard solutions of Fe(NH₄)₂(SO₄)₂.

RESULTS

Purification of coproporphyrinogenase. The highest enzyme specific activity (Table 1) was found in the mitochondrial fraction (mitochondria, grana, broken chloroplasts). The activity was several times higher than that found in the chloroplasts. However, as protoporphyrin is formed from δ-aminolaevulate or porphobilinogen by disrupted

chloroplasts from *Euglena* (Carell & Kahn, 1964), both chloroplasts and mitochondrial fractions were used for further purification.

The highest coproporphyrinogenase activity was found in the supernatant of the fraction treated with ammonium sulphate at 75% saturation (Table 2). The fractions saturated at 0–50% and 50–75% with respect to ammonium sulphate contained respectively only 6% and 35% of the specific activity compared with the supernatant fraction. The extract was purified by further adsorption with calcium phosphate gel, which removed 30–40% of the protein, resulting in a 42-fold purification.

On subsequent dialysis 1.6-fold activation of the enzyme was obtained with no change in the protein content. The removal of inhibitory substances such as ammonium sulphate seems possible. Extracts of coproporphyrinogenase (69-fold purification) retained their activity for weeks when stored frozen. Total activity as well as percentage yield was higher in the ammonium sulphate fraction than in the sonicated particulate fraction, which might also indicate the removal of inhibitor(s).

Effect of incubation time. Coproporphyrinogenase activity was linear with respect to time when the enzyme was incubated in the dark for up to 3 h, and

Table 1. Comparison of coproporphyrinogenase activity in different cellular fractions

The reaction mixture was incubated under the standard conditions described in the Materials and Methods section.

Fraction	10 ⁵ × Specific activity	Protein (μg/ml)	Endogenous iron (μg/ml)
Chloroplasts*	1.4	870	82
Mitochondria* (grana, broken chloroplasts)	8.3	180	21
Supernatant	0.6	270	11

* Soluble fraction after sonication and centrifugation.

Table 2. Purification of coproporphyrinogenase from *Nicotiana tabacum*

The reaction mixture was incubated under the standard conditions described in the Materials and Methods section.

Fractions	Total protein (mg)	10 ⁵ × total units	Yield (%)	10 ⁵ × Specific activity	Purification
Plant homogenate	1088	3800	100	3.5	1
Soluble fraction of mitochondria and chloroplasts after sonication	14	233	6	16.7	5
Supernatant after precipitation with 75%-satd. (NH ₄) ₂ SO ₄	3	327	9	109	31
Supernatant after calcium phosphate gel treatment	2	294	6	147	42
Supernatant after dialysis	2	480	12	240	69

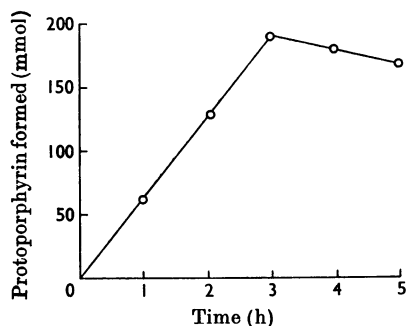


Fig. 1. Rate of coproporphyrinogenase reaction in relation to incubation time. The standard assay procedures were followed, with measurement of the formation of protoporphyrin IX. The assay mixture contained $30\mu\text{g}$ of protein and $0.3\mu\text{g}$ of iron.

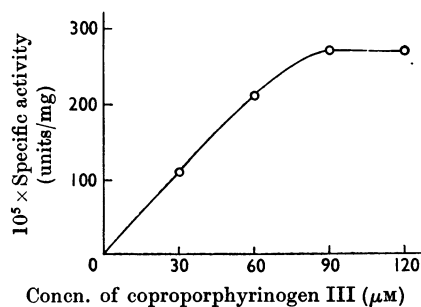


Fig. 3. Rate of coproporphyrinogenase reaction and substrate concentration. The standard assay procedures were used, with measurement of the formation of protoporphyrin IX. The assay mixture contained $25\mu\text{g}$ of protein with variations in concentration of coproporphyrinogen III as indicated. Iron content was $0.3\mu\text{g}/\text{ml}$ of solution.

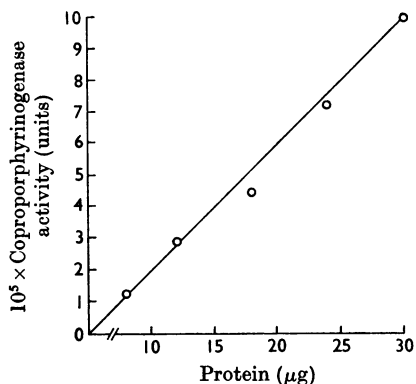


Fig. 2. Rate of coproporphyrinogenase reaction with respect to enzyme protein concentration. The standard assay procedures were used, with measurement of the formation of protoporphyrin IX. Iron content was $0.4\mu\text{g}/\text{ml}$ of solution.

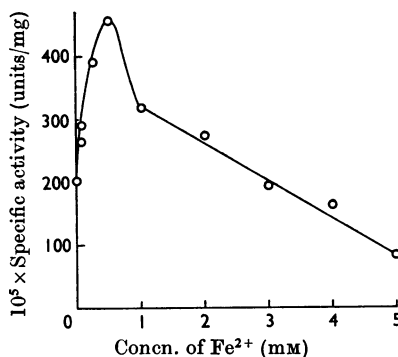


Fig. 4. Effect of Fe^{2+} on the activation of coproporphyrinogenase. The enzyme preparation ($30\mu\text{g}$ of protein/ml) was incubated under the standard conditions. Iron content in the control sample (no addition of Fe^{2+}) was $0.1\text{--}0.3\mu\text{g}/\text{ml}$.

decreased with longer incubation periods (Fig. 1). Reoxidation of coproporphyrinogen III probably occurred with a longer incubation time, resulting in lower substrate concentration and decreased enzyme activity.

Effect of enzyme concentration. Coproporphyrinogenase activity was proportional to the concentration of protein up to $30\mu\text{g}/\text{ml}$ (Fig. 2). The final concentrations of protein in the purified enzyme extract were below $30\mu\text{g}/\text{ml}$. Since 1 ml of extract was used in the assay this upper limit of protein concentration was never exceeded.

Effect of substrate concentration and the K_m value. The actual concentration of coproporphyrinogen III in the reaction mixture was not known, since

autoxidation takes place under conditions of the assay where aerobic conditions are required. Substrate saturation occurred at about $90\mu\text{M}$ (Fig. 3). The apparent K_m value estimated from Fig. 3 was about $3.6 \times 10^{-5}\text{M}$. This is in close agreement with values reported for preparations from other organisms (Batlle *et al.* 1965; Sano & Granick, 1961). This value is only approximate, however, because of the difficulty in determining the actual substrate concentration.

Effect of Fe^{2+} on coproporphyrinogenase. The effect of Fe^{2+} on the activation of the enzyme is shown in Fig. 4. Maximum activation (two-fold) was obtained with 0.5mM -ferrous ammonium

Table 3. *Effects of metal ions on coproporphyrinogenase activity*

The standard assay procedures described in the Materials and Methods section were used with concentrations of additives as indicated. The assay mixture contained 23 μg of protein/ml and 0.3 μg of endogenous iron/ml.

Additive	Concn. (mM)	$10^5 \times$ Specific activity	Activity (%)
None (control)		152	100
$\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$	5	64	42
	0.5	342	225
	0.1	214	141
FeCl_3	5	19	13
	0.5	70	47
	0.1	113	75
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	5	0	0
	0.5	76	50
	0.1	115	76
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	5	135	88
	0.5	186	120
	0.1	469	308
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	5	201	132
	0.5	247	162
	0.1	324	214

sulphate. Above this concentration a decrease in activity occurred. At 5mM- Fe^{2+} activity was inhibited 40% in comparison with the control. Although Fe^{2+} was not added to the control mixture, 0.1–0.3 μg of iron was present as a possible enzyme constituent.

Effects of other metal ions on coproporphyrinogenase. The effects of metal ions such as Fe^{3+} , Cu^{2+} , Co^{2+} and Mn^{2+} on coproporphyrinogenase activation were studied at three different concentrations (5, 0.5 and 0.1mM in the reaction mixture) and compared with that of Fe^{2+} (Table 3). The enzyme was inhibited by the addition of Fe^{3+} at the three concentrations studied; over 80% inhibition was found at a final concentration of 5mM. Cu^{2+} at 5mM inhibited enzyme activity completely. Co^{2+} and Mn^{2+} activated the enzyme three- and two-fold respectively at a lower concentration than did Fe^{2+} . Experiments with [^{14}C]coproporphyrinogen showed effects of metal ions on coproporphyrinogenase similar to those found by the spectrophotometric determinations with non-labelled substrates.

Effects of chelating agents on coproporphyrinogenase. Two chelating agents, *o*-phenanthroline and EDTA, at 1mM concentration inhibited enzyme activity. A 50% inhibition was found with EDTA, whereas only 10% was found with *o*-phenanthroline. The results support the idea that some metal ion is involved in coproporphyrinogenase activity.

DISCUSSION

The Michaelis constant, K_m , for coproporphyrinogenase was found by other investigators to be $3 \times 10^{-5}\text{M}$ (Batlle *et al.* 1965) and $2 \times 10^{-5}\text{M}$ (Sano & Granick, 1961). Our study with preparations from tobacco gave a value of $3.6 \times 10^{-5}\text{M}$, in close agreement with those reported by others. Aerobic conditions are required for the enzyme assay, resulting in continuous autoxidation of the substrate in spite of the presence of thioglycollate in the assay mixture. The solubility of coproporphyrinogen III in the reaction mixture at pH 7.4 is not known. The recovery of coproporphyrin III after reduction to coproporphyrin III by the sodium-amalgam method showed that coproporphyrin III might be partially destroyed during the reduction. All these factors make it difficult to carry out accurate kinetic studies under the standard assay conditions. Further, if any cofactors are required, K_m values measured without these factors would be in error. The involvement of cofactors such as CoA in the reaction is suggested by the fact that pantothenate-deficient cells of *Tetrahymena vorax* in the presence of adequate iron form only uroporphyrin III and coproporphyrin III from δ -aminolaevulate, whereas normal cells form protoporphyrin IX (Lascelles, 1957).

Metal ions such as Fe^{2+} , Co^{2+} and Mn^{2+} are possibly involved in the reaction. The highest activation effect on coproporphyrinogenase occurred at 0.5mM with Fe^{2+} and at concentrations at or below 0.1mM by Co^{2+} and Mn^{2+} . Absolute concentrations of Co^{2+} and Mn^{2+} that gave maximum activation of the enzyme were not determined.

Inhibition of coproporphyrinogenase by the addition of EDTA was much higher than that by *o*-phenanthroline, which has a higher affinity for Fe^{2+} . Coproporphyrinogenase was activated by Mn^{2+} and Co^{2+} at concentrations lower than where maximum activation occurred with Fe^{2+} . The metal associated with the enzyme *in vivo* could be Mn^{2+} or Co^{2+} or a combination of both.

The metal ion-chelate complex was possibly not fully developed under the incubation conditions. The Fe^{2+} -*o*-phenanthroline complex forms at an optimum pH range 5.5–6.5. A pH value other than the optimum could result in low affinity of *o*-phenanthroline to Fe^{2+} (Adler & George, 1965). Ferrous iron may also be bound to protein and chelating agents to some extent without enzyme inhibition.

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REFERENCES

- Adler, A. D. & George, P. (1965). *Analyt. Biochem.* **11**, 159.
- Axelrod, B. (1955). In *Methods in Enzymology*, vol. 1, p. 19. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Battle, A. M. del C., Benson, A. & Rimington, C. (1965). *Biochem. J.* **97**, 731.
- Cameron, B. F. (1965). *Analyt. Biochem.* **11**, 164.
- Carell, E. F. & Kahn, J. K. (1964). *Archs Biochem. Biophys.* **108**, 1.
- Carell, E. F. & Price, C. A. (1965). *Pl. Physiol., Lancaster*, **40**, 1.
- Colowick, C. P. (1955). In *Methods in Enzymology*, vol. 1, p. 98. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Falk, J. E. (1961). *J. Chromat.* **5**, 277.
- Gorham, P. R. (1955). In *Methods in Enzymology*, vol. 1, p. 22. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Granick, S. & Mauzerall, D. (1958). *Fedn Proc. Fedn Am. Socs exp. Biol.* **17**, 233.
- Gris, E. (1844). *C. r. hebd. Séanc. Acad. Sci., Paris*, **19**, 1118.
- Hsu, W. P. & Miller, G. W. (1965). *Biochim. biophys. Acta*, **111**, 393.
- Lascelles, J. (1956). *Biochem. J.* **62**, 78.
- Lascelles, J. (1957). *Biochem. J.* **66**, 65.
- Lascelles, J. (1962). In *The Bacteria*, vol. 3, p. 335. Ed. by Gunsalus, I. C. & Stanier, R. Y. New York: Academic Press Inc.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 256.
- Marsh, H. W., Evans, H. J. & Matrone, G. (1963a). *Pl. Physiol., Lancaster*, **38**, 632.
- Marsh, H. W., Evans, H. J. & Matrone, G. (1963b). *Pl. Physiol., Lancaster*, **38**, 638.
- Pappenheimer, A. J., jun. (1947). *J. biol. Chem.* **167**, 251.
- Read, R. R. & Lucarini, C. (1925). *Ind. Engng Chem. analyt. Edn*, **17**, 480.
- Rimington, C. & Sveinsson, S. L. (1950). *Scand. J. clin. Lab. Invest.* **2**, 209.
- Sano, S. & Granick, S. (1961). *J. biol. Chem.* **236**, 1173.
- Van Niel, C. B. (1944). *Bact. Rev.* **8**, 1.