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Studies on Ferrochelatase

THE ENZYMIC FORMATION OF HAEM IN PROPLASTIDS, CHLOROPLASTS AND PLANT MITOCHONDRIA

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1. Ferrochelatase was demonstrated in the chloroplasts and proplastids isolated from the primary leaves of beans (a dicotyledon) and oats (a monocotyledon). It was also detected in chloroplasts from etiolated bean seedlings made green by illumination before being harvested. The specific activities of the three types of bean organelles are similar, as are the specific activities of the oat proplastids and chloroplasts. 2. Chloroplasts from young spinach leaves also contain ferrochelatase; these chloroplasts were tested for their ability to form magnesium tetrapyrroles and found unable to catalyse the insertion of Mg^{2+} into mesoporphyrin IX. 3. Ferrochelatase was also detected in potato tuber mitochondria. 4. Ferrochelatase activity in these plant preparations is much less stable on storage than similar preparations from bacteria and animal tissues. 5. Temperature affects the activities of spinach chloroplast ferrochelatase and rat liver ferrochelatase differently. Activity of the chloroplast enzyme increases as the temperature rises from 20·6° to 26°, but becomes increasingly inactivated as the temperature rises further to 38°. The initial velocity of the mammalian enzyme, however, increases as the temperature rises from 25·8° to 65°, but the enzyme is inactivated after several minutes at 65°.

The enzyme ferrochelatase (protohaem ferrolyase, EC 4.99.1.1) catalyses the insertion of Fe^{2+} into a variety of dicarboxylic porphyrins to form the corresponding haems (Labbe, Hubbard & Caughey, 1963; Porra & Jones, 1963*a*,*b*,*c*). Haems are widely distributed in Nature as the prosthetic groups of haemoglobins, myoglobins, cytochromes, catalases and peroxidases. Consistent with this observation, ferrochelatase has been directly demonstrated in cell-free preparations from a wide variety of sources including many animal tissues, such as avian erythrocytes (Krueger, Melnick & Klein, 1956), and bone marrow (Kasuga, Konno & Sakai, 1954), reticulocytes (Clark & Walsh, 1959), liver, kidney and spleen (Lochhead & Goldberg, 1961) of mammals. This enzyme has been similarly demonstrated in two yeasts, *Saccharomyces cerevisiae* (Porra & Jones, 1963*b*,*c*) and *Torulopsus utilis* (Porra & Ross, 1965), in a wide variety of bacteria (Porra & Jones, 1963*b*,*c*) and in two photosynthetic bacteria, *Chromatium* strain D (Porra & Jones, 1963*b*,*c*) and *Rhodopseudomonas spheroides* (Porra & Lascelles, 1965).

Ferrochelatase is a particulate enzyme; it is located in the mitochondria of all the types of animal cells cited above (Sano, 1958; Rimington & Tooth, 1961; Lochhead & Goldberg, 1961) and also of yeast cells (Reithmüller & Tuppy, 1964). Ferrochelatase in bacterial cells is also a particulate enzyme (Porra & Jones, 1963*b*,*c*; Porra & Lascelles, 1965) and is presumably attached to cell membrane.

This paper demonstrates the presence of ferrochelatase activity in cell-free preparations from higher plants. A ferrochelatase was found in potato tubers and, like the animal and yeast enzymes, is located in the mitochondria. However, the enzyme was also found in the chloroplasts and proplastids isolated from the primary leaves of normal and etiolated bean and oat seedlings. Chloroplasts isolated from etiolated bean seedlings made green by subsequent exposure to light before being harvested also contain ferrochelatase; the specific activities of the enzyme in these chloroplasts and in the chloroplasts and proplastids of normal and etiolated bean seedlings are all similar. While this paper was in preparation a preliminary report (Jones, 1967) appeared describing the catalysis of the insertion of $^{59}Fe^{2+}$ into porphyrins by isolated chloroplasts.

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EXPERIMENTAL

Chemicals. Emasol 4130 (polyoxyethylene sorbitan mono-oleate) was a gift from the Kao Soap Co., Tokyo, Japan. Mesoporphyrin IX, prepared by the method of Baker, Ruccia & Corwin (1964) from haemin produced by the method of Labbe & Nishida (1957), was a gift from Dr J. E. Falk. The most satisfactory sample of GSH was supplied by Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; other samples gave rise to turbidity in reaction mixtures after several minutes' incubation at 38°.

Plant material. Potato tubers (*Solanum tuberosum* var. Kennebec and Brownell), freshly dug, were supplied by the Tasmanian Department of Agriculture at fortnightly intervals and stored at 15°. Spinach (*Spinacia oleracea*) plants were grown in liquid culture as described by Spencer & Possingham (1960) for tomato seedlings except that the spinach seeds were germinated and grown in vermiculite for 11 days before transfer into the nutrient solution of Tsui (1948). Leaves (1–2in. long) were harvested when the seedlings were 3–4 weeks old. Bean (*Phaseolus vulgaris* var. Brown Beauty) plants were grown in vermiculite at 25° for 11 days either in the light as described by Boardman & Anderson (1964) or in darkness, and others were removed from darkness after 10 days and illuminated for 16hr. at 400ft.-candles. Oat (*Avena sativa* var. Algerian) seedlings were grown at about 20° for 11 days in water-moistened vermiculite either under natural illumination or in darkness.

Preparation of cell-free fractions of plant tissues. (*a*) Spinach leaf fractions. A cell-free homogenate of spinach leaves (50g.) was prepared by the hand-chopping method of Spencer & Wildman (1964) in a buffer (100ml.) containing 0·4M-sucrose, 0·01M-KCl, 0·01M-$MgCl_2$, 4mM-2-mercaptoethanol and 0·05M-tris–HCl buffer, pH7·8; the crude homogenate was filtered through three layers of Miracloth (Chicopee Mills Inc., New York, N.Y., U.S.A.). Subsequent fractions were prepared at 4° in the same buffer. The filtered homogenate was centrifuged at 1000*g* for 10min. and the pellet was resuspended in buffer (final vol. 21ml.) with gentle stirring and shaking; 5ml. of this suspension was mixed vigorously in a Potter–Elvejhem homogenizer and designated the 'unwashed chloroplast fraction'. Much of the mitochondrial contamination was removed from 16ml. of this fraction by centrifuging again as above. The pellet was resuspended in fresh buffer (final vol. 16ml.) in a Potter–Elvejhem homogenizer and designated the 'washed chloroplast fraction'. The two supernatants from the above centrifugations were combined and centrifuged at 5000*g* for 10min. to remove many of the chloroplast fragments, and the pellet was discarded. The supernatant was centrifuged at 15000*g* for 20min. and the pellet resuspended in buffer (final vol. 5·5ml.) in a Potter–Elvejhem homogenizer and designated the 'mitochondrial fraction'; the 'supernatant fraction' (approx. 110ml.) was also retained for assay.

(*b*) Oat and bean primary-leaf fractions. The 'washed chloroplast fraction' and 'washed proplastid fraction' were prepared from the primary leaves of light-grown and etiolated seedlings respectively by the procedure used to prepare the washed chloroplast fraction from spinach leaves (see above).

(*c*) Potato tuber fractions. A cell-free homogenate of potato tuber was prepared by the method of G. G. Laties & T. Treffry (personal communication). Potato tuber (50g. block) was passed with buffer (100ml.) containing 0·25M-sucrose, 0·37M-mannitol, 4mM-cysteine and 2·5mM-tris–HCl buffer, pH7·8, through a juice extractor (G. Bauknecht G.m.b.H., Stuttgart, Germany); the extractor consists of a revolving stainless-steel toothed plate in the base of an aluminium-basket centrifuge (3000rev./min.; 750*g*) all encased in a plastic housing. To filter the homogenate the basket centrifuge was lined with moistened Miracloth. This procedure was repeated twice to yield 360ml. of homogenate. All subsequent fractions were prepared at 4° in the same buffer. One-third (120ml.) of the homogenate was centrifuged at 14000*g* for 20min. and the pellet resuspended (final vol. 5ml.) in a Potter–Elvejhem homogenizer and designated the 'crude mitochondrial fraction'. The remaining two-thirds of the homogenate (240ml.) was centrifuged at 1000*g* for 10min., thus precipitating much of the leucoplast contamination; the pellet was discarded and the supernatant centrifuged at 14000*g* for 20min. The pellet was resuspended (final vol. 10ml.) in a Potter–Elvejhem homogenizer and designated the 'purified mitochondrial fraction'.

Soluble rat liver ferrochelatase. This was prepared by the method of Porra, Vitols, Labbe & Newton (1967).

Enzyme assays. Ferrochelatase activity was measured as described by Porra *et al.* (1967) either by the pyridine haemochromogen method or by the continuous assay of porphyrin disappearance; a Cary model 14 spectrophotometer was used fitted with a Cary model 1471200 high-intensity light-source, a 0–0·1/0·1–0·2 *E* slide-wire and a Cary model 1462 scattered-transmission accessory. Cytochrome *c* oxidase activity was measured by the method of Smith (1955).

Other determinations. Protein concentration was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as standard. Total chlorophyll content of plant preparations was measured by the method of Arnon (1949).

RESULTS AND DISCUSSION

In the following experiments mesoporphyrin IX was used as substrate since it is more readily converted by ferrochelatase into mesohaem IX than is the natural substrate, protoporphyrin IX, into protohaem IX (Porra & Jones, 1963*b*,*c*). All the preparations of plant and animal origin were assayed on linear regions of their time–enzyme concentration curves; the activity of all preparations was destroyed by boiling.

Ferrochelatase in spinach chloroplasts. The data in Table 1 demonstrate the presence of ferrochelatase in carefully prepared spinach chloroplasts by the pyridine haemochromogen assay; the three experiments described are representative of the many performed. That the activity is located in the chloroplasts was demonstrated by the constant specific activity in the washed chloroplast and unwashed chloroplast fractions when expressed as mμmoles of mesohaem IX formed/hr./mg. of chlorophyll; microscopic examination revealed that the mitochondrial content of unwashed chloroplast fractions was small. This chloroplast activity was

Table 1. *Ferrochelatase in various spinach leaf fractions*

Fractions (0·5 and 1·0 ml.), prepared as described in the Experimental section, were incubated at 26° under N_2; under the assay conditions described mesohaem IX formation was linear for at least 90 min. The reaction mixture (final vol. 2·5 ml.) contained 100 mμmoles of mesoporphyrin IX, 0·25 ml. of ethanol, 0·25 ml. of 1% (w/v) Emasol 4130, 250 μmoles of tris–HCl buffer, pH 7·8, 10 μmoles of GSH and 100 mμmoles of $FeSO_4$, and the mesohaem IX formed was determined by the pyridine haemochromogen method (see Porra *et al.* 1967).

Expt. no.	Fraction	Ferrochelatase activity (mμmoles of mesohaem IX formed/hr./mg. of protein)	Ferrochelatase activity (mμmoles of mesohaem IX formed/hr./mg. of chlorophyll)
1	Unwashed chloroplasts	9·9	141
	Washed chloroplasts	9·0	112
2	Unwashed chloroplasts	3·3	60
	Washed chloroplasts	3·1	52
3	Unwashed chloroplasts	12·0	244
	Washed chloroplasts	15·0	254
	Mitochondrial	6·0	170
	Supernatant	0	0

found to be unstable; storage overnight at −15° resulted in a loss of approx. 60% of the original activity. This instability could explain the variation in activity observed in the different experiments of Table 1. A similar instability was observed with ferrochelatase activity in potato tuber mitochondria. Consequently the preparation and assay of all plant-cell organelles were performed as rapidly as possible.

Since the major tetrapyrrolic component of the chloroplasts is chlorophyll, the ability of the washed chloroplast fraction to insert Mg^{2+} into mesoporphyrin IX was also investigated. Washed chloroplast fraction was incubated as described for the ferrochelatase assay (see Table 1), but magnesium chloride was substituted for ferrous sulphate. The reaction mixture, treated with iodoacetamide, pyridine and alkali as described by Porra *et al.* (1967), was examined with a spectrofluorimeter capable of recording absolute emission and excitation spectra, designed in these Laboratories by Mr S. W. Thorne. In alkaline pyridine solution mesoporphyrin IX has emission bands at 621 and 685 mμ and magnesium mesoporphyrin IX bands at 583 and 645 mμ. As the latter bands could not be detected it was concluded that no Mg^{2+} insertion had occurred; a similar negative finding was obtained by Jones (1967).

Ferrochelatase activity was also demonstrated in the mitochondrial fraction from spinach leaves; however, it is not possible to obtain leaf-cell mitochondria free of chloroplast fragments. As the specific activity of this fraction, expressed per mg. of chlorophyll, was less than that observed in the chloroplast fractions (Table 1), it is possible that the activity is associated with the chloroplast fragments and not with the mitochondria.

Ferrochelatase in potato tuber mitochondria. To ascertain whether plant mitochondria, like those of animal and yeast cells, contain ferrochelatase a non-green plant tissue was examined. Plant mitochondria can be prepared from potato tubers and gross contamination with leucoplasts avoided (see the Experimental section). The results in Table 2 indicate the presence of ferrochelatase in these mitochondria; ferrochelatase activity (*A*), expressed as mμmoles of mesohaem IX formed/hr./mg. of protein, increased during purification of the mitochondria. However, in Expts. 1 and 2 (Table 2) the activity (*B*) of cytochrome *c* oxidase, chosen as a mitochondrial marker, did not increase during purification. This anomaly may be due to the adsorption on the precipitated leucoplasts of mitochondrial fragments with high oxidase but little ferrochelatase activity; indeed, this explanation is supported by the fact that the discarded leucoplast pellet from Expt. 2 (Table 2) contained 25% of the cytochrome *c* oxidase activity of the crude mitochondrial fraction but scarcely detectable ferrochelatase activity. By adding this lost cytochrome *c* oxidase activity to that of the purified mitochondrial fraction, but without adjusting the protein content, corrected values (shown in parentheses; Table 2) were obtained for the cytochrome *c* oxidase activity and for the ferrochelatase/cytochrome *c* activity ratio (*A*/*B*); the latter value for the *A*/*B* ratio was little different from that for the crude mitochondrial fraction. A low *A*/*B* ratio would be expected of mitochondrial fragments compared with intact mitochondria since the fragments, having no

Table 2. *Ferrochelatase and cytochrome* c *oxidase activities in mitochondrial preparations from potato tubers*

Fractions were prepared and assayed for cytochrome *c* oxidase activity as described in the Experimental section. The fractions (0·5 and 1·0 ml.) were incubated at 26° under N_2 and assayed for ferrochelatase as described in Table 1; under these conditions mesohaem IX formation was linear for at least 60 min. The values in parentheses are defined in the text.

Expt. no.	Fraction	Ferrochelatase activity (*A*) (mμ moles of mesohaem IX/hr./mg. of protein)	Cytochrome *c* oxidase activity (*B*) (first-order rate constant/sec./mg. of protein)	*A*/*B* ratio
1	Crude mitochondria	1·28	0·05	25·6
	Purified mitochondria	1·66	0·04	41·5
2	Crude mitochondria	1·10	0·04	27·5
	Purified mitochondria	1·50	0·04	37·5
			(0·05)	(30·0)
3	Crude mitochondria	2·9	0·05	58·0
	Purified mitochondria	3·5	0·06	58·3

permeability barrier against substrates of the cytochrome *c* oxidase assay, would exhibit high cytochrome *c* oxidase values; however, ferrochelatase is always measured in the presence of detergent and ethanol so that permeability problems do not arise. In Expt. 3 (Table 2) better separation was achieved and no detectable ferrochelatase or cytochrome *c* oxidase activity was found in the discarded leucoplast pellet; again the *A*/*B* ratios of crude mitochondrial and purified mitochondrial fractions were almost identical. Thus it would appear that most, if not all, of the ferrochelatase activity in the crude mitochondrial fractions is associated with the mitochondria.

The adsorption of cytochrome *c* oxidase activity on the leucoplasts was typical of the many experiments performed even though, on some occasions, attempts were made to regain this activity by washing the leucoplast pellet; the complete separation achieved in Expt. 3 (Table 2) was unique. These results indicate a strong tendency for mitochondrial fragments to adhere to leucoplasts.

Ferrochelatase activity in proplastids and chloroplasts of higher plants. To determine whether proplastids from etiolated plant tissues contain ferrochelatase, spinach was discarded as a source material in favour of beans (*Phaseolus vulgaris*) because the large seed reserves of the latter will support substantial dark growth. Washed chloroplast and washed proplastid fractions isolated from the primary leaves of normal and etiolated bean seedlings respectively exhibited ferrochelatase activity. The specific activities, expressed as mμmoles of mesohaem IX formed/hr./mg. of protein, were similar: 3·4 for chloroplasts and 3·0 for proplastids. Chloroplasts isolated from etiolated bean plants made green by exposure to light for 16 hr. also contained ferrochelatase and a similar specific activity (2·8) was again obtained.

Bean chloroplasts contain cytochromes *f*, b_6 and 559 whereas proplastids lack the cytochrome 559 component; the latter appears during greening and spectra indicate an increase in total cytochrome (Boardman, 1968). The fact that ferrochelatase activities in proplastids and chloroplasts are similar suggests that this enzyme is not a factor in regulating haemoprotein synthesis in the developing chloroplast. In bacteria, also, the specific activity of ferrochelatase remains constant under conditions that cause an increase in cellular haem content (Porra & Lascelles, 1965).

To ascertain whether the proplastids and chloroplasts of a monocotyledon also contain ferrochelatase both normal and etiolated oat seedlings were grown. Washed chloroplast and washed proplastid fractions isolated from the primary leaves contained the enzyme; as with the bean organelles the specific activities in the chloroplasts and proplastids were similar, namely 2·5 and 2·8 mμmoles of mesohaem IX formed/hr./mg. of protein.

Response of ferrochelatase activity to temperature. There have been several reports that haems are formed spontaneously and rapidly when ferrous salts are mixed with porphyrins at 37° near pH 7 (Heikel, Lockwood & Rimington, 1958; Granick & Mauzerall, 1958; Tokunaga & Sano, 1966), thus casting doubt on the existence of a truly enzymic Fe^{2+}-incorporation reaction. However, Lowe & Phillips (1962) found that the non-enzymic rate of haem formation is 200-fold lower than the enzymic rate even in the presence of sodium dodecyl sulphate and 8-hydroxyquinoline, which greatly stimulate metalloporphyrin formation.

Observations on the effect of temperature on ferrochelatase activity in spinach chloroplasts and in a rat liver mitochondrial preparation support the contention that Fe^{2+} incorporation is an enzymic process. This activity in chloroplasts was less stable

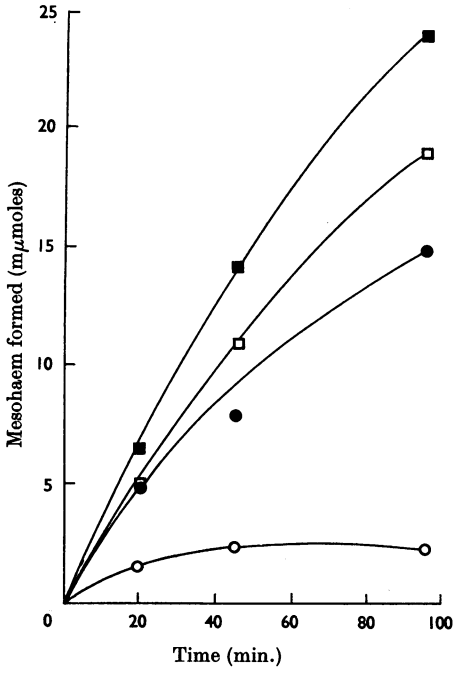

Fig. 1. Response of spinach chloroplast ferrochelatase activity to temperature. Washed chloroplast fraction (1·0ml.) was incubated under N_2 and assayed for ferrochelatase activity as described in Table 1 at 20·6° (●), 26° (■), 30° (□) and 38° (○).

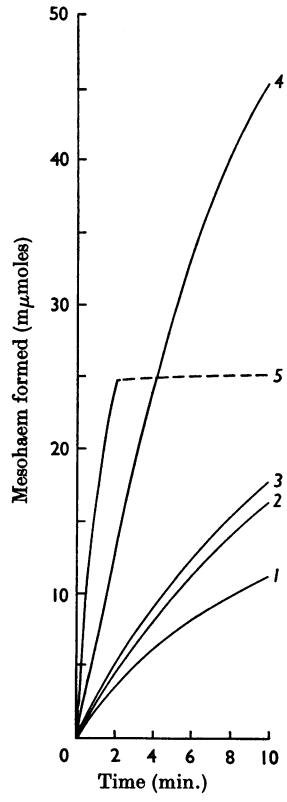

Fig. 2. Response of rat liver ferrochelatase activity to temperature. Soluble rat liver ferrochelatase preparation (0·75ml.) was assayed by the continuous assay of porphyrin disappearance (Porra *et al.* 1967). Traces *1*, *2*, *3*, *4* and *5* were observed at 25·8°, 37°, 44·2°, 52·5° and 65°. The broken line in trace *5* is the mean of the trace where the pen-noise level reached ± 0·005 *E*, on the 0–0·1/0·1–0·2 *E* slide-wire, owing to turbidity caused by protein denaturation. It was confirmed by the pyridine haemochromogen method that mesohaem IX formation did not exceed approx. 25mμmoles at 65°.

at high temperatures (Fig. 1) than in the preparation from rat liver mitochondria (Fig. 2). Both the continuous spectrophotometric assay of porphyrin disappearance (Fig. 2) and the pyridine haemochromogen assay were used with the rat liver preparation; with the latter assay and incubation periods up to 1hr. the results of Fig. 2 were confirmed. Only the pyridine haemochromogen assay was used with chloroplasts because flocculation during incubation interfered with the continuous assay; the precipitate, however, was dispersed in alkaline pyridine.

The different temperature responses shown in Figs. 1 and 2 are not consistent with a common, spontaneous, mechanism for haem formation and, together with the evidence cited above of a slow non-enzymic rate of haem formation, suggest that the two extracts contain enzymes with different thermal stabilities. The enzymic nature of the reaction is also supported by the demonstration that the activity is linearly dependent on protein concentration, is destroyed by boiling and is non-diffusible on dialysis (Porra & Jones, 1963*a*,*c*). The reaction is inhibited by thiol reagents (Porra & Ross, 1965), and chromatography on Sephadex G-100 revealed that the active component from yeast has a molecular weight of approx. 100000 (Reithmüller & Tuppy, 1964).

Significance of ferrochelatase in chloroplasts and proplastids. Since chloroplasts were found to possess their own species of ribosomes (Lyttleton, 1962) and characteristic DNA (Ris & Plaut, 1962) the possibility that these organelles possess a limited degree of autonomy has been discussed in considerable detail (Gibor & Granick, 1964; Granick & Gibor, 1967; Whitfeld & Spencer, 1968). Chloroplasts have been shown to carry out a great number of biochemical activities. In addition to a variety of reactions associated with photosynthesis, chloroplasts are known to have a capacity for DNA

synthesis (Spencer & Whitfeld, 1967*a*), DNA-dependent RNA synthesis (Semal, Spencer, Kim & Wildman, 1964; Kirk, 1964; Spencer & Whitfeld, 1967*b*), protein synthesis (Spencer, 1965) and fatty acid synthesis (Brooks & Stumpf, 1966). The presence in plant chloroplasts and especially in plant proplastids of the enzyme that converts porphyrins into haems is consistent with the presence of haemoproteins in these organelles. Though the disrupted algal chloroplasts of *Euglena gracilis* (Carell & Kahn, 1964) possess enzymes capable of converting δ-aminolaevulic acid into protoporphyrin IX, the plant chloroplast converts this precursor only as far as porphobilinogen (Granick, 1961); however, the plant proplastid (Granick, 1961) can convert δ-aminolaevulic acid via protoporphyrin IX into magnesium tetrapyrroles of the chlorophyll pathway. Since protoporphyrin IX is the natural substrate of ferrochelatase, the presence of this enzyme in plant proplastids makes them competent to synthesize not only chlorophyll but also haem from simple precursors, thus indicating a further degree of biochemical autonomy for the plant chloroplast especially in the proplastid stage of its development. Moreover, since the location of ferrochelatase in plant tissues is not limited to the mitochondrion, as in animal and yeast cells, it now seems possible to eliminate the cumbersome alternative that the prosthetic groups of chloroplast haemoproteins are produced in the mitochondrion and then transported to the chloroplast. Finally, Smillie, Graham, Dwyer, Grieve & Tobin (1967) have demonstrated in *Euglena gracilis* that the syntheses of the chloroplast cytochromes 552 and 562 are chloramphenicol-sensitive and have suggested that the protein moieties of these chloroplast haemoproteins are also synthesized within the chloroplast by the chloramphenicol-sensitive chloroplast ribosomes.

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