Haem Pigments of Cytoplasmic Particles from Non-Photosynthetic Plant Tissues

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Keilin (1925) first described the cytochromes in a wide variety of whole tissues from animals and plants. Yakushiji (1935) and Okunuki (1939) later studied the distribution of several cytochromes in higher plants, and, in particular, in algae, from which Yakushiji (1935) extracted cytochrome *c*. Mann (1938) found that the haematin of non-photosynthetic plant tissues is largely concentrated in the meristematic zones. From the disrupted cotyledons of germinating *Phaseolus* seeds, Hill & Bhagvat (1939) and Bhagvat & Hill (1951) sedimented particles which contained a cytochrome system (cytochromes *a*, *b* and *c*) analogous to that found in heart muscle and yeast by Keilin (1925). Hill & Scarisbrick (1951) extended this work and described cytochrome *f*, which occurs only in chloroplasts. Another haem protein, called cytochrome b_3, was found by these workers in the press juice of macerated leaves and in non-photosynthetic tissues. Hill (1954) found cytochrome b_6 in chloroplasts from etiolated barley. Lundegårdh (1954*a*, *b*) has observed in bundles of wheat roots yet another pigment, cytochrome *dh*, which he considers to be identical with plant succinic dehydrogenase. Both Vernon & Kamen (1954) and Chance & Smith (1955) have described a number of haem pigments occurring in photosynthetic bacteria.

The haem proteins occurring in plant tissues are thus fairly well described in terms of their spectroscopic properties, but there is much less information concerning their localization and function in plant cells. Cytochrome *f* (Davenport, 1952; Davenport & Hill, 1952; Lundegårdh, 1954*a*) and cytochrome b_6 (Hill, 1954) both probably act as hydrogen acceptors in the photosynthetic pathway for oxidation of the reduced products formed during the photolysis of water by chloroplasts. The complex of cytochromes *a*, *b* and *c* observed by Bhagvat & Hill (1951) is typical of mitochondria and sarcosomes from animal tissues in which these haem pigments function as hydrogen acceptors during oxidation of succinic acid (see Chance, 1952; Keilin & Slater, 1953; Morton, 1955*a*). Martin & Morton (1955) found that microsomes isolated from beet petioles contained a pigment apparently identical with cytochrome b_3 of Hill & Scarisbrick (1951), and showed that this haem protein is associated with a cyanide-insensitive pathway for enzymic oxidation of reduced di- and tri-phosphopyridine nucleotides. In this paper, the term cytochrome b_3 (M. & M.) refers to the microsomal cytochrome of Martin & Morton (1955), and the term cytochrome b_3 (H. & S.) to the pigment observed by Hill & Scarisbrick (1951).

This paper reports further studies of the haem compounds of plants, particularly of the cytochromes associated with cytoplasmic particles. Whereas cytochrome *b* is associated with the mitochondria, cytochrome b_3 (M. & M.) is almost entirely localized in the microsomal particles. Cytochrome *b* is intimately associated with the succinic dehydrogenase system of plants. Cytochrome *dh* has not been detected in enzymically active mitochondria isolated from developing wheat roots and other plant tissues.

MATERIALS AND METHODS

Reduced diphosphopyridine nucleotide (*DPNH*). This was obtained from C. F. Boehringer and Soehne, Mannheim, Germany.

Sodium dithionite. Laboratory reagent grade $Na_2S_2O_4$ (British Drug Houses Ltd.) was used.

Ethylenediaminetetraacetic acid (*EDTA*). This was used as the disodium salt (Versene) obtained from British Drug Houses Ltd.

Pyridine. Analytical-grade reagent was redistilled over pellets of NaOH, only the initial distillate and a small coloured residue being rejected.

Nitrogen gas. Cylinders were supplied by British Oxygen Co. Ltd. as containing not more than 0·002% (v/v) of O_2.

Spectroscopy. For general qualitative investigations, a low-dispersion (Sorby–Browning type) microspectroscope was used. This instrument contains a prism that produces two incident light beams, thus permitting direct comparison of two spectra. This replaced the eyepiece of a standard laboratory microscope. For measurement of the wavelengths of absorption bands, the low-dispersion spectroscope was replaced by a Hartridge reversion type. The spectroscopes were calibrated against the 546 mμ. mercury line and frequently checked against a solution of reduced cytochrome *c* (α-band, 550 mμ.) from heart muscle. For quantitative estimation of the concentration of reduced haemochromogen, a Zeiss comparator microspectroscope was used.

Absorption spectrophotometry. A Beckman spectrophotometer (Model DU) was used. The wavelength scale between

226 and 656 mμ. was checked against known lines of the emission spectrum from a mercury-discharge lamp. In no case did the error exceed the tolerances given by the manufacturers (*Beckman Instruction Manual* 305, 1954, p. 8). Quartz cuvettes of either 0·5 or 1 cm. light path were used.

Reflectance spectrophotometry. The preparations, packed in crushed ice, were transported to Sydney, N.S.W., by air. Measurements were made at the National Standards Laboratory, C.S.I.R.O., Sydney, within 4–12 hr. after isolation of the particles. The paste was placed in a 0·25 cm. × 2·5 cm. diameter optical cell and the percentage reflectance transmittance determined between 400 and 700 mμ. with a General Electric self-recording Hardy-type spectrophotometer, and a band-width of about 10 mμ. A didymium standard was used for wavelength calibration.

Standard protein haemochromogen. This was prepared from a suspension of washed bovine red cells by heating to 60° with 0·1 N-NaOH, cooling and adding sufficient solid $Na_2S_2O_4$ to reduce the haemochromogen completely. For the pyridine haemochromogen, pyridine (25%, v/v) was added immediately before the $Na_2S_2O_4$. The concentrations of both solutions were estimated from the extinctions at 558 mμ., molecular extinction coefficients of $32{\cdot}5 \times 10^3$ being used in both cases.

Estimation of the concentration of protohaematin. The intensity of the broad α-band of the reduced pyridine protohaemochromogen of the preparation was compared visually with that of a suitable standard (see Elliott & Keilin, 1934; Hill & Hartree, 1953). The plant preparation was made alkaline with 0·1 N-NaOH, and pyridine (approx. 0·2 vol.) and a trace of $Na_2S_2O_4$ were added. The preparation was immediately transferred to a 1 cm. optical cell, which was sealed from air with a thin cover-slip. The intensity obtained with the standard preparation of reduced pyridine protohaemochromogen (see above) was then matched to that of the unknown by adjustment of the length of the light path, with a comparator microspectroscope.

Nitrogen. This was estimated by a micro-Kjeldahl procedure (Morton, 1955*b*).

Preparation of dispersions of cytoplasmic particles

The procedure used for the preparation of cytoplasmic particles varied slightly for each different tissue, being based on experience gained in the preparation of cytoplasmic fractions from beet petiole as described previously (Martin & Morton, 1956*a*). The choice of dispersion medium used previously (0·2 M sucrose in 0·2 M potassium phosphate buffer, pH 7·4) was largely determined by the requirement of minimum interference in chemical studies. These considerations did not apply to studies on the cytochrome constitution of the isolated fractions. The medium used contained sucrose (0·2 M), potassium phosphate buffer, pH 7·4 (0·1 M), potassium succinate (0·1 M), EDTA (0·01%, w/v) and cysteine hydrochloride (0·1%, w/v, added immediately before the disruption of the tissue). The medium was designed to keep the cytochromes largely in the reduced state in which they may be more stable (see Morton, 1955*c*), and to inhibit the activity of polyphenol oxidases. The tissue dispersions were prepared as described below. All operations after the collection of the tissue were carried out in a cold room at approx. 2°.

Silver-beet petiole. Young silver-beet plants were freshly harvested and the white petioles dissected free of leaf laminae and washed thoroughly with cold water. The petioles were chopped by hand into small pieces (approx. 0·5 cm. × 0·5 cm. × 0·1 cm.) and ground for about 2 min. with the medium (150 ml./100 g. of chopped tissue) in a large earthenware mortar previously cooled to about 0°.

Wheat roots. These were prepared from wheat germinated for about 48 hr., kindly supplied by Barrett's Food Company Pty. Ltd., Melbourne. The roots were removed by vigorous rubbing of the wheat on a coarse stainless-steel screen (8 holes/in.). Approx. 20 g. of material, consisting mostly of roots with some intermixed cotyledons (see Martin & Morton, 1956*b*), was obtained from each kg. of germinating wheat. About 100 g. of the tissue was then ground by hand in a pre-chilled mortar with 25 g. of washed sand and 200 ml. of dispersion medium.

Onion apical stem. From eight to ten germinating onion bulbs, the apical stem bases (approx. 25 g. of tissue) were dissected and chopped into small pieces. They were ground in a chilled mortar with 50 ml. of dispersion medium (as above but containing 0·2%, w/v, of cysteine hydrochloride).

'Arum' spadices. The flowers were rubbed from the spadices from five white 'arum lilies' (*Zantedeschia aethiopica*). Approx. 20 g. of the flowers was ground with 50 ml. of dispersion medium.

Fractionation of dispersions. After grinding, each dispersion was filtered through four thicknesses of muslin cheese cloth. Fractionation of these dispersions was carried out as described previously (Martin & Morton, 1956*a*).

In some cases during preliminary studies, the intermediate fraction was not separated. In most cases the sedimented particles were washed by suspending in 0·15 M-NaCl and recentrifuging as appropriate for the particular fraction (see Martin & Morton, 1956*a*). In this way the total material for any fraction was obtained in a single centrifuge cup, a distinct advantage for spectroscopic studies.

In addition to the above fractions, a 'total particle' preparation was sometimes isolated by centrifuging the tissue dispersion at 50000 *g* for 90 min.

EXPERIMENTAL AND RESULTS

Spectroscopic and spectrophotometric studies of whole plant tissues and isolated cytoplasmic particles

Whole tissues

Visual observations. Suitable thicknesses of the tissue were immersed for 15 min. in mM-KCN in potassium phosphate buffer (0·05 M), at pH 7·4, in order to inhibit the cytochrome oxidase activity. The absorption bands of the cytochromes so reduced were then observed with a microspectroscope. Petioles of silver beet (*Beta vulgaris*), apical stems of onion, spadices of 'arum lily' (*Z. aethiopica*) and bundles of wheat roots showed essentially the same absorption spectrum. A broad band was visible, extending from about 552 to 566 mμ., with a midpoint at about 556 mμ. This band could not be adequately resolved into its components. A weak band was usually visible at about 600 mμ., together with a third, even weaker band at about 530 mμ.

No absorption bands at 571 mμ. [or at 585 mμ. in the presence of KCN (Lundegårdh, 1954*b*)] were observed in wheat roots.

Fraction obtained at 1500 *g for* 15 *min.*

Visual observations. With some tissues, such as silver-beet petiole, an absorption band at 675 mμ. (attributed to chlorophyll) could be seen in this material. However, no other absorption bands could be detected.

Mitochondria

Visual observations. Mitochondria were examined as a firm pellet obtained after centrifuging. When reduced with $Na_2S_2O_4$, mitochondria from silver-beet petioles, onion apical stems and wheat roots showed essentially similar absorption spectra. Although the bands could be easily resolved with the low-dispersion microspectroscope, with the Hartridge reversion spectroscope only the edges of the bands could be properly measured. The strong absorption band in the green extended from 552–555 mμ. to 564 mμ. This is probably the fused α-bands of cytochromes of the *c* type (α-bands between 550 and 554 mμ.) and cytochrome *b* (α-band 562–564 mμ.). A band was also seen at 603–604 mμ. This is considered to be the composite α-band of cytochromes *a* and a_3.

When a few crystals of sodium succinate and a few drops of 0·01 M-KCN in potassium phosphate buffer (0·2 M, pH 7·4) were added to a pellet of freshly isolated mitochondria from wheat roots, the absorption bands of the several reduced cytochromes appeared. The spectrum was directly compared with that of cytochrome *c* from heart muscle with the low-dispersion spectroscope. Whereas the extreme edge of the α-band of the *c*-type cytochrome corresponded to the position of reduced heart-muscle cytochrome *c* (at 550 mμ.), the mean position of this α-band was estimated to be at about 552–554 mμ. The α-band of the *c*-type component could be quite well resolved and distinguished from the broader cytochrome *b* component (α-band at about 562 mμ.).

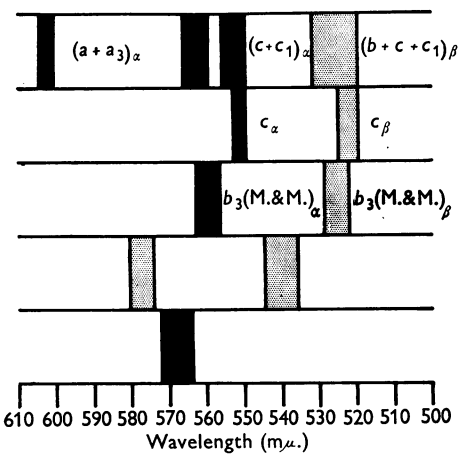

Fig. 1. Diagrammatic representation of visible absorption bands of plant haem pigments. *A*, Wheat-root mitochondria with sodium succinate and KCN; *B*, wheat-root mitochondria heated to 70°, with $Na_2S_2O_4$; *C*, wheat-root microsomes with DPNH (anaerobically), or with $Na_2S_2O_4$ (aerobically); *D*, precipitated supernatant proteins from silver-beet petiole, oxygenated; *E*, precipitated supernatant proteins from silver-beet petiole, with $Na_2S_2O_4$.

When the wheat-root mitochondria were heated at 70° for 30 min. and then reduced with $Na_2S_2O_4$, there appeared only a faint, narrow band, corresponding exactly with the position of the band of reduced cytochrome *c* from heart muscle. The same band appeared (together with a faint band at about 600 mμ.) when solid ascorbic acid was added to an unbuffered pellet of wheat-root mitochondria. Thus it appears that these mitochondria contain two cytochromes of the *c* type, cytochrome *c* with an α-band at 550 mμ., and considerably more cytochrome c_1 with an α-band at about 554 mμ. (see Discussion). The relative positions of the observed bands are shown diagrammatically in Fig. 1.

Addition of solid DPNH, together with buffered KCN (as above) caused appearance of bands at about 553 mμ., 562 mμ. (faint but definite) and at about 600 mμ., corresponding to the α-bands of cytochromes of the *c*, *b* and *a* types.

When a preparation of wheat-root mitochondria was reduced with $Na_2S_2O_4$, and KCN (about mM) was added subsequently to the pellet, there was no appreciable change in the absorption bands except for that at 604 mμ. This band appeared to shift toward the blue end of the spectrum. This was due to a widening of the band, so that the mean position of the broadened band was at 599 mμ. A similar widening of the band at 604 mμ. occurred after bubbling coal gas (used as a source of carbon monoxide) through a heavy suspension of wheat-root mitochondria. These observations suggest the presence of both cytochromes *a* and a_3 in wheat-root mitochondria.

A number of mitochondrial preparations from wheat roots have been examined as described above and also following treatment with liquid air as described by Keilin & Hartree (1949, 1955). No bands corresponding to those of the pigment called cytochrome *dh* by Lundegårdh (1954*b*) (α-band at about 571 mμ., shifting to about 585 mμ. with KCN) have so far been observed in the preparations reduced with either sodium succinate or $Na_2S_2O_4$.

Spectrophotometric studies. The absorption spectrum of the mitochondria from wheat roots was determined as described by Shibata, Benson & Calvin (1954) and by a method similar to that of Holton (1955) and Keilin & Hartree (1955). Some measurements were also made with clear dispersions

in sodium deoxycholate (cf. Strittmatter & Ball, 1952). However, most satisfactory results were obtained with a firmly packed pellet of the material placed so as to adhere to the side of a 0·5 cm. cuvette. The absorption of this pellet was read against a blank consisting of a mitochondrial or microsomal preparation in which the cytochromes had been destroyed by heating at 60° with 0·1 vol. of 30 % (w/v) H_2O_2. The concentration of this blank was adjusted so that an extinction of approx. 0·3 was obtained at 600 mμ. Fig. 2 shows the spectrum of mitochondria from beet petiole when reduced with $Na_2S_2O_4$. The maxima appear at 605, 558, 525 and 425 mμ.

Fig. 3 shows the reflectance spectrum of wheat-root mitochondria, both oxidized and reduced with $Na_2S_2O_4$. In the reduced preparation, maxima occur at 602, 556, 582 and 422 mμ. The difference spectrum (reduced minus oxidized) for this preparation is shown in Fig. 4. When plotted in this way, the maxima are seen at 603, 558, 526, 484 and 427 mμ. The difference spectrum (not shown) of wheat-root mitochondria, when oxidized and subsequently reduced with sodium succinate, has maxima at similar positions. The nature of the pigments responsible for these maxima is considered in the Discussion.

Intermediate fraction

On reduction with $Na_2S_2O_4$, this fraction invariably showed an absorption spectrum essentially the same as that of microsomes, except for an occasional weak band at about 605 mμ. This fraction was not systematically examined.

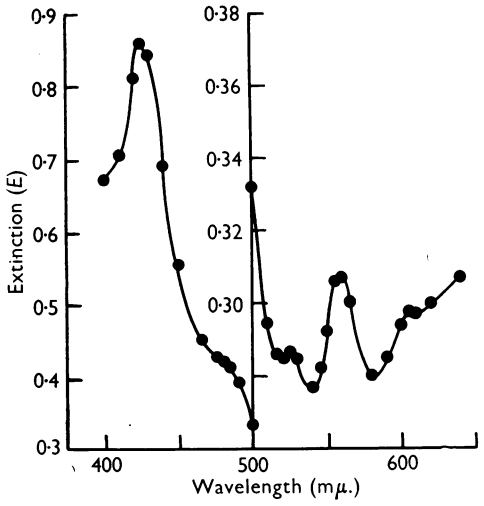

Fig. 2. Absorption spectrum of a firmly packed pellet of mitochondria from silver-beet petiole reduced with $Na_2S_2O_4$, and determined in the Beckman spectrophotometer as described in the text.

Microsomes

Spectrophotometric studies. Some of the properties of the cytochromes associated with plant microsomes have been described previously (Martin & Morton, 1955). The reduced pigment of beet-petiole microsomes had absorption maxima at 559·5, 525 and 425 mμ. The enzymic reduction of this cytochrome after addition of DPNH under anaerobic conditions, and the very rapid oxidation of the reduced cytochrome on exposure to air, were observed spectroscopically.

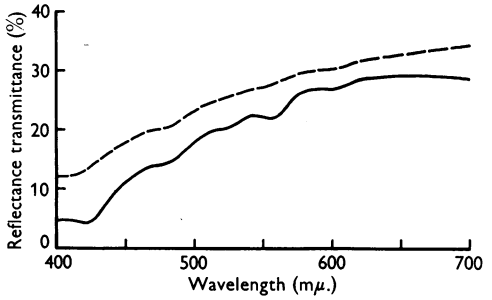

Fig. 3. Reflectance spectra of wheat-root mitochondria as determined with the General Electric self-recording spectrophotometer. Oxidized, – – –; reduced with $Na_2S_2O_4$, ——.

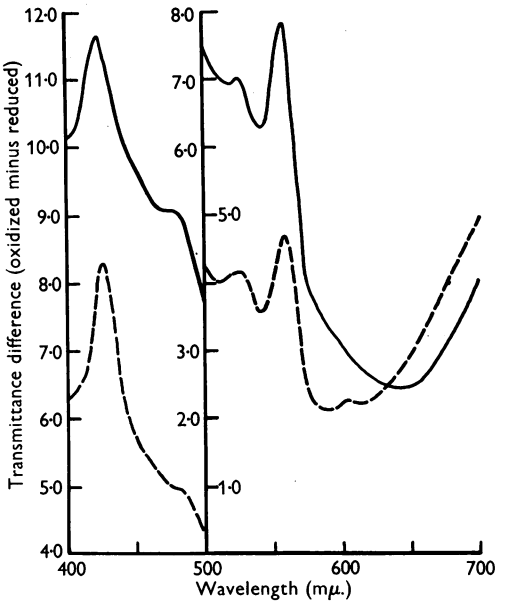

Fig. 4. Difference spectra (reduced with $Na_2S_2O_4$, minus oxidized) for mitochondria (– – –) and microsomes (——) from wheat root. Values were obtained by difference of the measurements obtained with the General Electric recording spectrophotometer as shown in Fig. 3 (mitochondria) and Fig. 6 (microsomes).

In further experiments, the anaerobic reduction of this cytochrome has been demonstrated spectrophotometrically as follows. A thick suspension of beet-petiole microsomes in 0·1 M potassium phosphate buffer, pH 7·4, was aerated thoroughly with oxygen and placed in a 1 cm. cuvette fused to the bottom of a Thunberg-type tube. The absorption spectrum between 500 and 600 mμ. of the oxidized microsomes was read against a blank treated with H_2O_2 (see above). The tube was then evacuated and the atmosphere replaced with oxygen-free nitrogen.

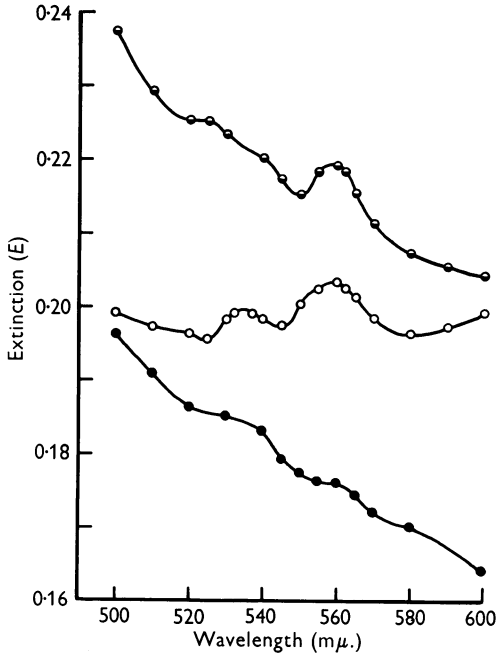

Fig. 5. Absorption spectra of a firmly packed pellet of microsomes from beet petiole, determined in the Beckman spectrophotometer as described in the text. Oxidized, ●; reduced with DPNH under anaerobic conditions, ◒; reduced with $Na_2S_2O_4$, ○.

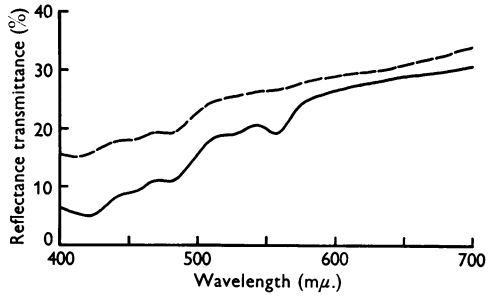

Fig. 6. Reflectance spectra of wheat-root microsomes as determined with the General Electric recording spectrophotometer. Oxidized, ---; reduced with $Na_2S_2O_4$, ——.

The process was repeated three times. A small amount of solid DPNH from the side arm was tipped into the cuvette and mixed by agitation, and the absorption spectrum redetermined after incubation for 15 min. at room temperature. Finally, solid $Na_2S_2O_4$ was added to the cuvette and the absorption spectrum again determined. As shown in Fig. 5, the pigment was substantially reduced by DPNH under anaerobic conditions.

Fig. 6 shows the reflectance spectrum of a preparation of wheat-root microsomes, both oxidized, and reduced with $Na_2S_2O_4$. The maxima of the reduced preparation appear at 558, 526, 480 and 422 mμ. The difference spectrum for the preparation appears in Fig. 4, which shows maxima at 558, 525, 480 and 425 mμ. Measurements made with a firm pellet in the Beckman spectrophotometer as described for mitochondria gave maxima in the reduced preparation at 558, 525, 483 and 425 mμ., in substantial agreement with the results obtained with the General Electric instrument.

Localization of cytochrome b_3 (*M. & M.*) *in microsomes.* Cytochrome b_3 (M. & M.) has not been detected in either the precipitate obtained at 1500 *g* for 15 min. or in the supernatant fraction, but if present in the mitochondria it would be masked by the strong absorption bands of reduced cytochromes at 552–555 mμ. and at 563 mμ. Mitochondria and microsomes were therefore separately incubated anaerobically with sodium succinate (0·05 M) in the presence of 0·1 M potassium phosphate buffer, pH 7·4, and mM-KCN. After incubation at 37° for 30 min., the intensities of the absorption bands were compared with a protein haemochromogen preparation of known concentration. The preparations were then fully reduced with $Na_2S_2O_4$ and the cytochrome concentrations estimated as before. The result of one such experiment is shown in Table 1.

Table 1. *Estimation of haemochromogens in beet-petiole mitochondria and microsomes*

Mitochondria and microsomes were prepared by centrifuging dispersions of silver-beet petiole as described in the text. The haemochromogens were estimated after anaerobic incubation with 0·05 M sodium succinate at pH 7·4 in the presence of mM-KCN, and subsequently after reduction with $Na_2S_2O_4$. Measurements were made by comparing the intensity of the α-band of the reduced cytochromes with that of a standard solution of protein haemochromogen, prepared as described under Methods. Results are expressed as μg. of haematin/100 g. fresh wt.

	Haemochromogen formed when reduced with	
Cytoplasmic fraction	Sodium succinate	$Na_2S_2O_4$
10000 *g*, 15 min. (mitochondria)	3·00	3·00
50000 *g*, 90 min. (microsomes)	0	2·55

A preparation of microsomes from beet petiole was washed once with 0·15 M-NaCl and then assayed for DPNH–cytochrome *c* reductase activity as described previously (Martin & Morton, 1956*a*). It had an activity of 70 μmoles of cytochrome *c* reduced/hr./mg. of N at 20°. The haematin concentration in this preparation was estimated as $5{\cdot}3 \times 10^{-3}$ μmole of haematin/mg. of N (2·9 μg. of haematin/100 g. fresh wt.). From these figures, a value of 13 000 μmoles of cytochrome *c* reduced/hr./μmole of cytochrome b_3 (M. & M.) haematin is obtained. In other preparations, slightly higher activities/μmole of haematin have been found (see Martin & Morton, 1955).

Final supernatant

Visual observations. In order to concentrate any haem pigments in the soluble fraction remaining after centrifuging a beet-petiole dispersion at 50 000 *g* for 90 min., the supernatant was dialysed (in Visking cellulose tubing) against saturated ammonium sulphate at 0° for 18 hr. The precipitate was collected by centrifuging at about 2000 *g* for 20 min. at 0° and examined under the low-dispersion microspectroscope.

The preparation showed no distinct absorption bands. Several different portions of the wet precipitate were then treated as follows.

When $Na_2S_2O_4$ was added, an intense broad band appeared, extending from 565 to 570 mμ., with a mid-point at about 568 mμ. No other absorption band was observed (see Fig. 1). When coal gas (used as a source of carbon monoxide) was bubbled through this preparation (reduced with $Na_2S_2O_4$), the absorption band was shifted by about 4 mμ. toward the red end of the spectrum, and a weak band appeared at about 536 mμ. Addition of KCN (mM) to another portion of precipitate (reduced with $Na_2S_2O_4$) also appeared to shift the absorption band somewhat toward a longer wavelength.

The reduced pyridine haemochromogen formed with another portion of the precipitate showed very intense bands at 557 and 530 mμ., whereas the reduced imidazole haemochromogen showed bands at 562 and 530 mμ.

When oxygen was bubbled through another portion of precipitate, faint bands appeared at about 578 and 540 mμ. (see Fig. 1). These bands slowly disappeared on standing. No absorption bands could be observed after the subsequent addition of potassium ferricyanide at pH 5·0. No pigment could be detected in an ether extract of a portion of the precipitate. However, when a further portion was treated with 0·5 N-HCl for 2 hr. at room temperature, and the ether extract evaporated to dryness, a pigment was obtained which formed a reduced pyridine haemochromogen similar to that formed from protohaemin.

Distribution of haem compounds among the cytoplasmic fractions from beet petiole

Table 2 shows the distribution of haem compounds among the cytoplasmic fractions from beet petiole. The 'total dispersion' figures were obtained by adding the results for a 'total particle' preparation to those for the precipitate obtained on dialysis of the supernatant fraction (in Visking cellulose tubing) against saturated ammonium sulphate. Only 84 % of the total haematin of the dispersion was recovered.

DISCUSSION

The special difficulties associated with spectrophotometry of turbid materials, such as suspensions of cytoplasmic particles, have recently been investigated by several workers (see Shibata *et al.* 1954; Holton, 1955; Keilin & Hartree, 1955). The difference spectra (reduced minus oxidized compounds) are particularly valuable for differentiating compounds which may not be adequately resolved

Table 2. *Distribution of haem compounds in cytoplasmic particles from silver-beet petiole*

Particulate fractions were prepared from silver-beet petiole dispersions (see text). The haem compounds were estimated by forming the pyridine haemochromogen as described under Methods. Analyses for the total dispersion were obtained by adding the results for a 'total particle' preparation (see text) to those for the supernatant. Individual determinations are shown in parentheses below the mean values.

Cytoplasmic fraction	Haematin (μg./100 g. fresh wt.)	N (mg./100 g. fresh wt.)	Haematin (μmoles/g. of N)	Percentage of total dispersion
Total dispersion	32·6	39·1	1·22	100
1500 *g*, 15 min. (debris, nuclei)	0·7 (0·6, 0·7)	3·2 (3·3, 3·1)	0·28	2
10 000 *g*, 15 min. (mitochondria)	4·29 (4·22, 4·45, 4·20)	2·95 (3·0, 2·9)	2·14	13
50 000 *g*, 90 min. (microsomes)	2·59 (2·70, 2·55, 2·52)	2·45 (2·6, 2·3)	1·55	8
Supernatant	19·9 (19·8, 20·0)	30·5 (31·6, 29·3)	1·00	61
Recovery (%)	—	—	—	84

in the spectra of the reduced cytoplasmic particles. In the present investigation with the Beckman instrument, losses due to scattering of light by the particles have been reduced by using a thin layer of packed material. As an alternative approach, use has been made of reflectance spectrophotometry, theoretical aspects of which are discussed by Giovanelli (in preparation). Moss (1956) has used this method for quantitative assay of the cytochromes of bacteria. The method is quite sensitive, a paste of bacteria containing 1 μg. of protohaemin/g. dry wt. showing a change of 0·06 % reflectance transmittance at 560 mμ. (Moss, 1956).

In general, the results obtained with both the above procedures showed good agreement with visual observations made with a low-dispersion microspectroscope. Because of the great sensitivity of the latter method, especially when used with particles brought to the temperature of liquid air as described by Keilin & Hartree (1949, 1955), it has been used as the method of choice for establishing the presence (or absence) of any cytochrome component. Owing to the relatively large bandwidths of the commercial spectrophotometers, cytochromes with closely adjacent absorption peaks may not be adequately resolved with these instruments (see Keilin & Hartree, 1955).

Cytochromes of plant mitochondria

Cytochromes a *and* a_3. Bhagvat & Hill (1951) showed that particles (presumably mitochondria) sedimented from disrupted plant tissues contained cytochrome *a*. Plant mitochondria also show considerable cytochrome oxidase activity (Hill & Bhagvat, 1939; Bhagvat & Hill, 1951; Millerd & Bonner, 1953; Martin & Morton, 1956*a*). Since Keilin & Hartree (1939) have shown that cytochrome oxidase of animal tissues is probably identical with cytochrome a_3 of animal tissues, it would be expected that cytochrome a_3 would occur in plant mitochondria. The effects of potassium cyanide and carbon monoxide on the absorption band at 604 mμ. suggest that cytochrome a_3 occurs in wheat-root mitochondria. This is supported by Lundegårdh's (1951, 1952) spectrophotometric detection of the γ-band of an *a*-type cytochrome in bundles of wheat roots. Cytochrome *a*, in contrast to cytochrome a_3, has a relatively weak Soret band (Keilin & Hartree, 1939; Lemberg & Legge, 1949, p. 369), so that its detection in whole wheat roots would be unlikely. Lundegårdh (1952) also observed a 'shift' of the bands of the ferrocytochrome in the presence of either carbon monoxide or potassium cyanide. The failure to detect the Soret band of the *a*-type cytochromes in the absorption spectra of the mitochondria from wheat roots and from beet petioles is probably due to the comparatively low concentration of cytochrome a_3 in the plant mitochondria, and to the relative insensitivity of the spectrophotometric methods in this region of the spectrum. Clearly there is a need for a more detailed study of the cytochromes of the *a*-type of plant mitochondria.

Cytochromes b *and* dh. As shown in Figs. 2–4, the α-bands of the haem pigments of reduced mitochondria cannot be resolved spectrophotometrically (cf. also, for example, Holton, 1955). However, they may be readily differentiated with a low-dispersion spectroscope. The maxima shown in Figs. 2–4 are therefore considered to be the resultant of several different cytochromes, of which cytochrome *b* is predominant. The maxima of reduced cytochrome *b* of plant mitochondria probably occur at about 563, 529 and 427 mμ. Although cytochrome b_6 of Hill (1954) has similar spectroscopic properties, this pigment apparently occurs only in chloroplasts and it is improbable that it occurs also in plant mitochondria. Moreover, the cytochrome *b*-type pigment of wheat-root mitochondria is fairly readily denatured by acetone. This differentiates it from cytochrome b_6, which is more stable (Hill, 1954).

Lundegårdh (1954*b*) has described a pigment called 'cytochrome *dh*', observed in bundles of wheat roots. The difference spectrum of this cytochrome shows a maximum at about 571 mμ., shifting to about 585 mμ. in the presence of potassium cyanide. If the pigment is identical with succinic dehydrogenase, as suggested by Lundegårdh (1954*b*), then it should be found in wheat-root mitochondria, which have a high succinic dehydrogenase activity (Martin & Morton, 1956*b*). It has been shown that succinic dehydrogenase is localized in the mitochondria in other plant tissues (Martin & Morton, 1956*a*). However, no such pigment has yet been observed spectroscopically either in the mitochondria or in other fractions isolated from developing wheat roots. Improvement of the sensitivity by freezing the preparations at the temperature of liquid air (Keilin & Hartree, 1949, 1955) has not enabled detection of this pigment. Lundegårdh (1954*a*, *b*) found that cytochrome *dh* occurred at about the same concentration as cytochrome *b* in whole wheat roots. The spectrum of wheat-root mitochondria fails to show any pigment corresponding to cytochrome *dh*, although cytochrome *b* is very prominent (Figs. 2 and 3). A possible explanation of this discrepancy is that very young (about 48-hr.-old) roots were used in the present investigation, whereas apparently quite mature roots (from plants 2–3 weeks old) have been used in Lundegårdh's studies (see Lundegårdh, 1951). Unfortunately, the exact nature of the material used is not stated in the later paper (Lundegårdh, 1954*b*). It is of interest that Lundegårdh (1952) observed the pigment with an

absorption maximum at about 482 mμ. which is present in wheat-root mitochondria (Figs. 3, 4) and wheat-root microsomes (Figs. 4, 6) as well as in beet-petiole microsomes (Martin & Morton, 1955).

Cytochrome *b* of plant mitochondria appears to bear a relationship to the plant succinic dehydrogenase system similar to that which cytochrome *b* of heart muscle bears to the succinic dehydrogenase system in this tissue. In the latter, cytochrome *b* apparently acts as a hydrogen carrier in the respiratory pathway and is quite distinct from succinic dehydrogenase itself. This enzyme, which was separated from cytochrome *b* by butanol treatment (Morton, 1950), has been shown to be a flavoprotein (see Morton, 1955*a*).

The succinic dehydrogenase system of plants appears to resemble closely that of animal tissues (Martin & Morton, 1956*a*), although it is somewhat more labile (Price & Thimann, 1951; Martin & Morton, 1956*a*, *b*). When mitochondria from beet or wheat roots are held at room temperature for several hours, the succinic dehydrogenase activity may decline very considerably without any apparent change in the intensity or position of the α-band of reduced cytochrome *b*. Thus it seems very likely that cytochrome *b* of plant mitochondria is quite distinct from succinic dehydrogenase, which is probably a flavoprotein as in animal tissues.

Cytochromes c *and* c_1. Goddard (1944) showed that cytochrome *c* isolated from wheat germ had essentially the same absorption spectrum and other properties as the similar pigment from heart muscle. Hill & Scarisbrick (1951) obtained cytochrome *c* (α-band, 550 mμ.) and cytochrome b_3 (H. & S.) (α-band, 559 mμ.) in solution in autolysates of several plant tissues. Cytochrome *c* has also been directly observed in wheat-root mitochondria (see Results), but in no other fraction from wheat roots. Cytochrome *c* was obtained in solution by autolysis of a beet-petiole dispersion (see Martin & Morton, 1955). There is therefore little doubt that all the plant tissues studied contain cytochrome *c*, which is entirely localized in the mitochondria.

However, when reduced with either sodium succinate or sodium dithionite, mitochondria from wheat roots and silver-beet petioles show a band at 554–555 mμ., rather than at 550 mμ. as might be expected. This band is unlikely to be due to cytochrome *f* (α-band, 555 mμ. when reduced), since this cytochrome has not been demonstrated to occur elsewhere than in chloroplasts of photosynthetic tissues (Davenport, 1952; Davenport & Hill, 1952). It seems more probable that this pigment is cytochrome c_1, first described by Yakushiji & Okunuki (1940) and recently shown by Keilin & Hartree (1955) to be identical with cytochrome *e* of Keilin & Hartree (1949). Cytochrome c_1 of heart muscle, when reduced, shows bands at 553, 524 and 418 mμ. (Keilin & Hartree, 1955; Estabrook, 1955). Keilin & Hartree (1955) found that the absorption maxima of the complex of cytochromes *c* and c_1 is at approx. 552 mμ. in heart muscle, in which the concentration of cytochrome *c* is in excess of that of cytochrome *b*. In plant mitochondria it seems likely that the absorption maximum of a mixture of cytochromes *c* and c_1 would occur at a longer wavelength, owing to the relatively low concentration of cytochrome *c* and the relatively high concentration of cytochrome *b*. Thus an absorption maximum of 553–554 mμ. could well be explained by the occurrence of cytochrome c_1 in the presence of cytochromes *b* and *c*. Visual observations (see Results) suggest that the concentration of cytochrome c_1 in wheat-root mitochondria is rather greater than that of cytochrome *c*.

Cytochrome of plant microsomes

The spectrophotometric studies described here (see Results and Figs. 4–6) confirm the earlier observations of Martin & Morton (1955) and extend them to microsomes from wheat roots. The possible function of cytochrome b_3 (M. & M.) in the DPNH–cytochrome *c* reductase and DPNH–oxidase activities of microsomes has been discussed previously (Martin & Morton, 1955, 1956*a*).

The evidence which suggests that the microsomal cytochrome is similar to a pigment observed by Hill & Scarisbrick (1951) has been published (Martin & Morton, 1955). However, as yet neither pigment has been extensively purified, so that their identity cannot be established beyond doubt. In order to avoid possible confusion, it now seems desirable to differentiate the pigments observed by these different authors in the manner suggested in this paper.

Neither the fraction obtained at 1500 *g* for 15 min. (containing plastids and nuclei) nor the final supernatant shows any absorption band in the 555–560 mμ. region when reduced with sodium dithionite. Whereas the cytochromes of mitochondria are completely reduced by anaerobic incubation with sodium succinate in the presence of potassium cyanide, cytochrome b_3 is not so reduced (Table 1). Hence plant mitochondria must contain negligible amounts of cytochrome b_3 (M. & M.), which appears to be entirely localized in the microsome fraction.

Pigments of the supernatant fraction

Table 2 shows that a considerable portion of the haem compounds of beet petiole remain in the supernatant fraction after centrifuging at 50 000 *g* for 90 min. The nature of these haem compounds is by no means clear. Catalase and peroxidase possibly comprise a small portion, as in other plant tissues (see Hill & Hartree, 1953; Lundegårdh, 1954*b*).

The appearance of bands at about 578 and 540 mμ. on oxygenation of the precipitate, followed by the slow disappearance of the bands (either under anaerobic conditions or in air), resembles somewhat the observations made on haemoglobins of legume-root nodules (Keilin & Wang, 1945), of protozoa (Keilin & Ryley, 1953), of baker's yeast (Keilin, 1953) and of some moulds (Keilin & Tissières, 1953). All these non-mammalian pigments form oxygenated ferrous complexes resembling mammalian oxyhaemoglobin. No ferric compound similar to 'acid' methaemoglobin was detected on oxidation of the precipitated proteins with potassium ferricyanide, but this may be due to the relatively low molecular extinction coefficient of the ferric compound (see Lemberg & Legge, 1949, p. 228). Keilin & Tissières (1953) and Keilin & Ryley (1953) likewise failed to detect any absorption bands after treatment of the mould and protozoan material with potassium ferricyanide. For the same reasons, the bands observed on oxygenation of the precipitated proteins are unlikely to be those of a ferric compound such as denatured protein parahaematin. The fading of the bands on cessation of oxygenation is probably due to the reducing activity of material (including traces of cysteine) in the precipitated proteins.

The compound which shows the two absorption bands on oxygenation of the precipitated proteins was observed in several different batches prepared as described, but the bands failed to appear when the proteins were precipitated at room temperature instead of at 0°. This suggests that the compound is rather labile.

The broad, strong absorption band which appeared at about 568 mμ. on addition of sodium dithionite to the precipitated proteins may be due to a denatured protein haemochromogen. This is indicated by the effects of added carbon monoxide and of added potassium cyanide, which probably form the carbon monoxide haemochromogen and the cyanide ferroporphyrin respectively. Denatured globin carbon monoxide haemochromogen shows bands at 571 and 545 mμ., and denatured globin cyanide ferroporphyrin at 568 and 540 mμ. (Lemberg & Legge, 1949, p. 228). These correspond rather closely with the positions of the observed bands (see Results).

It seems possible, therefore, that the extracts of beet petioles contain, in very small amount, a haem compound capable of forming an oxygenated ferrous complex. Most of the haematin of the supernatant fraction, however, appears to occur as a complex with denatured protein. This may be due to denaturation of haem compounds during the fractionation of the tissue dispersion and subsequent precipitation with ammonium sulphate. If so, the compounds are particularly labile.

SUMMARY

1. The haem compounds of white petioles of silver beet, roots of germinating wheat, flowers from spadices of 'arum lilies' (*Zantedeschia aethiopica*) and apical stems of onion, have been studied. Pieces of tissue, tissue dispersions and fractions separated by differential centrifuging were used.

2. Low-dispersion microspectroscopes, a Beckman spectrophotometer and a General Electric self-recording Hardy-type spectrophotometer were used. The quantitative distribution of the haem compounds among the cytoplasmic particles of beet petiole has been determined.

3. The plant mitochondria contain cytochromes closely resembling in absorption spectra cytochromes *a*, *b*, *c* and c_1 of animal tissues. Cytochrome *b* is predominant. Cytochrome *dh* has not been detected in mitochondria from wheat roots or from silver-beet petioles. There is evidence that cytochrome a_3 occurs in wheat-root mitochondria.

4. Cytochromes *b*, *c* and c_1 of plant mitochondria are reduced enzymically on addition of sodium succinate, and on addition of reduced diphosphopyridine nucleotide. Cytochrome *b* appears to be intimately concerned in the succinic dehydrogenase system of plants. The nature of plant succinic dehydrogenase is briefly discussed.

5. A haemprotein, here called cytochrome b_3 (M. & M.), is localized almost entirely in the microsome fraction, and is reduced enzymically by the addition of reduced diphosphopyridine nucleotide.

6. Spectroscopic observations showed that the supernatant fraction obtained from beet petiole contained in very small amount a labile pigment capable of forming an oxygenated ferrous complex. However, most of the haematin in this fraction occurred as a complex with denatured protein.

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Levels of Oxidized and Reduced Diphosphopyridine Nucleotide and Triphosphopyridine Nucleotide in Tumours

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It is probable that the availability of pyridine nucleotides as well as the relative proportions of the oxidized and reduced forms of these nucleotides are among the factors controlling the metabolic behaviour of cells. For this reason, previously published work (Glock & McLean, 1955*a*) on the distribution of oxidized and reduced diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) in animal tissues has now been extended to a variety of tumours. This supplements the recent investigation of Jedeikin & Weinhouse (1955), who determined the contents of oxidized and reduced diphosphopyridine nucleotide (DPN^+ and DPNH respectively) of several tumours by a spectrophotometric method employing alcohol dehydrogenase and appropriate substrates. Besides supplying additional data on the DPN^+ and DPNH contents of various tumours, the present investigation provides the first reliable figures for TPN contents. Oxidized and reduced pyridine nucleotides have been determined by extremely sensitive methods (Glock & McLean, 1955*b*) which involve coupling enzymically reduced pyridine nucleotides with their respective cytochrome *c* reductases and then following the rate of reduction of cytochrome *c* spectrophotometrically.

EXPERIMENTAL

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