THE RESPIRATORY CHAIN OF BEETROOT MITOCHONDRIA

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Summary

Mitochondria were isolated from root tissue of red beetroot (*Beta vulgaris* L.) and the components of the respiratory chain for oxidation of succinate and of reduced diphosphopyridine nucleotide (DPNH) were studied. Succinate, DPNH, ferrocytochrome *c*, and malate were used as substrates, and 2,6-dichlorophenol-indophenol, ferricytochrome *c*, and oxygen as hydrogen (electron) acceptors. DPNH was oxidized without addition of cytochrome *c* and malate without addition of DPN. These observations suggest that the respiratory chain was retained intact in the isolated mitochondria. Cytochromes *b*, c_1, and *c* were identified spectroscopically by the positions of their characteristic α-absorption bands. The very small amount of cytochrome *c* present may indicate some loss of this component during isolation of the mitochondria. An absorption band near 600 mμ was attributed to cytochromes $(a+a_3)$.

Succinate–cytochrome *c* reductase was strongly inhibited by antimycin A and somewhat less by 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO), 2-hydroxy-3-(2-methyloctyl)-1,4-naphthoquinone (SN 5949), and phenylurethane. These results suggest that there is only one pathway for hydrogen transport between succinate and cytochrome *c*. In contrast, the DPNH–cytochrome *c* reductase was incompletely inhibited by antimycin A, HOQNO, and SN 5949. Amytal strongly inhibited this system. However, antimycin A and amytal were less inhibitory when tested with the DPN-stimulated oxidation of malate. The results suggest that these are alternative pathways for oxidation of DPNH.

The rates of oxidation of succinate, DPNH, and of ferrocytochrome *c* were all stimulated by freezing and thawing or by hypotonic treatment of the freshly isolated mitochondria. With malate and with succinate as substrates, P/O ratios of 0·8 and 2·0 respectively were obtained. The results have been compared with those obtained with mitochondria from animal and other plant tissue.

I. Introduction

It has been shown that mitochondria, isolated from a wide variety of plant and animal tissues, are the site of aerobic respiration and that there is a definite sequence of hydrogen and electron carriers involved in the oxidation of substrates (see Chance and Williams 1956). Although studies on plant mitochondria have been less extensive, it appears that a number of the respiratory components are similar to those of animal mitochondria. However, it cannot be assumed that the respiratory pathways in plant and animal mitochondria are identical.

Known properties of cytochrome components of plant tissues have been reviewed by Hill and Hartree (1953), Hartree (1957), Morton (1958), and Smith

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and Chance (1958). The latter workers have drawn attention to some of the conflicting observations by different workers.

Martin and Morton (1956*a*, 1956*b*, 1957) carried out enzymic, spectroscopic, and spectrophotometric studies to determine the functions and properties of cytochromes of mitochondria and microsomes isolated from silver-beet petioles and from wheat roots. Spectrophotometric observations of the cytochromes of particles from wheat roots by Lundegårdh (1958) are in general agreement with the results of Martin and Morton (1957) except for the localization of some of the pigments. Particles from cauliflower buds (Crane 1957) and tobacco roots (Sisler and Evans 1959) have been used for similar studies.

Honda, Robertson, and Gregory (1958) studied the effect of salts on the oxidation of reduced diphosphopyridine nucleotide and of reduced cytochrome *c* by beetroot mitochondria, but did not determine the components of the oxidation systems. This paper describes studies to determine the sequence of reactions in the respiratory chain of beetroot mitochondria.

II. Materials and Methods

(*a*) *Preparation of Mitochondrial Suspension*

The mitochondria were isolated from red beet (*Beta vulgaris* L.) obtained commercially. The tissue (200 g), diced into cubes of approx. 0·5 cm, was blended for 30 sec in a medium (220 ml) of 0·4M sucrose, containing 0·045M tris(hydroxymethyl)aminomethane (TRIS) and 0·005M ethylenediaminetetra-acetic acid (EDTA). The homogenate was strained through a double layer of muslin and centrifuged at 1500 *g* for 10 min. The supernatant was again strained and then centrifuged at 6000 *g* for 30 min. The precipitate was suspended in 0·4M sucrose and centrifuged at 20,000 *g*. The mitochondria were washed at least once to remove the red pigment and the final suspension was made up to a volume of 10 ml. All operations were carried out in the cold (about 1°C) with chilled solutions and apparatus. Centrifuging was carried out at −3°C. The suspension was kept in an ice-bath during the course of an experiment.

(*b*) *Measurement of Enzymic Activities*

The general procedures have been described by Martin and Morton (1956*a*). A Beckman spectrophotometer, model DU, with photomultiplier attachment was used in a room maintained at 25°C. All reaction mixtures were made up to a final volume of 3 ml in cuvettes with 1 cm light path. The reactions were started by the addition of the mitochondrial suspension and reaction rates were estimated from the changes in optical density (O.D.) at the appropriate wavelengths. Molar extinction coefficients of $6{\cdot}22\times10^3$ at 340 mμ for reduced diphosphopyridine nucleotide, of $19{\cdot}6\times10^3$ (reduced minus oxidized compound) at 550 mμ for cytochrome *c*, and $19{\cdot}1\times10^3$ at 600 mμ for 2,6-dichlorophenolindophenol, were used for estimation of these compounds.

The reaction mixtures contained 31 mM TRIS–28 mM acetic acid buffer, at pH 7·2. Where used, inhibitors were added to the reaction mixture before addition of enzyme.

All activities are expressed as the change in optical density (Δ O.D.)/hr/mg mitochondrial nitrogen, as estimated at the appropriate wavelength for the first 30 sec of reaction.

Oxidative phosphorylation was determined essentially as described by Hunter (1955) with air as the gas phase. The manometer vessel contained 0·27M sucrose, 31 mM TRIS, 28 mM acetic acid, 2 μM cytochrome *c*, 13 mM potassium phosphate, 1 mM adenosine diphosphate, 6·7 mM $MgCl_2$, 10 mM NaF, 20 mM glucose, approx. 1 mg of hexokinase, and 370 μg mitochondrial nitrogen per reaction mixture (3 ml). Oxygen uptake was measured at 25°C for about 30 min. The reaction was then stopped with 5 per cent. perchloric acid and glucose 6-phosphate formed enzymically was estimated spectrophotometrically with glucose 6-phosphate dehydrogenase and triphosphopyridine nucleotide, after neutralization.

Attempts to estimate oxidative phosphorylation with DPNH by direct spectrophotometry according to Pullman and Racker (1956) were unsuccessful because of the high adenylate kinase activity. About 50 per cent. inhibition of this adenylate kinase activity was obtained with sodium fluoride (0·01M).

(*c*) *Estimation of Nitrogen*

Samples were digested in concentrated sulphuric acid with mercury as catalyst. Distillations and titrations were carried out essentially as described by McKenzie and Wallace (1954).

(*d*) *Chemicals*

Diphosphopyridine nucleotide (DPN) and reduced diphosphopyridine nucleotide (DPNH) were obtained from C. F. Boehringer and Soehne, Mannheim, Germany. Adenosine diphosphate (ADP) and triphosphopyridine nucleotide (TPN) were obtained from Pabst Laboratories, Wisconsin, U.S.A. Hexokinase and glucose 6-phosphate dehydrogenase were supplied by Sigma Chemical Co., St. Louis, U.S.A. Cytochrome *c* was prepared according to the method of Keilin and Hartree as modified by Potter (1951), and then dialysed against distilled water. Reduced cytochrome *c* was prepared by reduction with sodium dithionite ($Na_2S_2O_4$), the excess dithionite being oxidized with a stream of air.

Amytal (5-ethyl-5-*iso*amylbarbiturate, sodium salt) was obtained from Eli Lilly and Co. Ltd. Other inhibitors were gifts from various workers. 2-Hydroxy-3-(2-methyloctyl)-1,4-naphthoquinone (SN 5949) and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO) were made up in 1 per cent. NaOH, and antimycin A and phenylurethane in 40 per cent. (v/v) ethanol.

III. Results

Some of the factors affecting the rates of aerobic oxidation of both DPNH and of reduced cytochrome *c* by beet mitochondria have been described by Honda, Robertson, and Gregory (1958). In the present work a set of conditions was chosen and used for all enzymic reactions. Seasonal variations in enzymic activity occurred (cf. Martin and Morton 1956*a*) but the rate of aerobic oxidation of DPNH was used as a reference for comparison of different preparations.

(*a*) *DPNH Oxidase Activity*

The rate of aerobic oxidation of DPNH was proportional to the amount of mitochondria used. With a concentration of 0·1 mM DPNH and with 40–70 μg of mitochondrial nitrogen as generally used, the rate of oxidation of DPNH was linear for at least 10 min.

Since the addition of 0·1 mM potassium cyanide completely inhibited aerobic oxidation of DPNH it is probable that cytochrome *c* oxidase acts as the terminal oxidase for this reaction. As shown in Table 1, the rate was stimulated by addition of heart-muscle cytochrome *c*, and this increased the apparent effect of inhibitors.

TABLE 1

EFFECT OF VARIOUS INHIBITORS ON THE DPNH OXIDASE ACTIVITY OF BEETROOT MITOCHONDRIA WITH AND WITHOUT ADDED CYTOCHROME *c*

Reaction mixtures, at pH 7·2, were equilibrated with air and contained 0·27M sucrose, 0·13 mM DPNH, 31 mM TRIS, 28 mM acetic acid, and 59 μg of mitochondrial nitrogen per reaction mixture (3 ml). In experiment 2, heart-muscle cytochrome *c* (2 μM) was added but this was omitted in experiment 1

Expt. No.	Control Rate (Δ O.D./hr/mg N at 340 mμ)	Percentage Inhibition with:					
		Antimycin A (44 μg)	Phenyl-urethane (2·7 mM)	Ethanol (2·7%, v/v)	Amytal (3·6 mM)	HOQNO (25 μg)	SN 5949 (25 μg)
1	27	57	43	—	46	—	—
2	95	80	69	29	77	65	62

Of the compounds investigated, antimycin A was the most inhibitory. Ethanol, in which both the antimycin A and the phenylurethane were dissolved, caused much less inhibition than either (Table 1).

(*b*) *Cytochrome* c *Oxidase Activity*

The aerobic oxidation of reduced heart-muscle cytochrome *c* (60 μM) was completely inhibited by 0·1 mM potassium cyanide. There was a slight but variable inhibition with ethanol (2·7 per cent. v/v). There was no inhibition by antimycin A, phenylurethane, amytal, HOQNO, or SN 5949 when used at similar concentrations to those shown in Table 1, at which there is considerable inhibition of DPNH oxidase activity.

(*c*) *Cytochrome* c *Reductase Activity*

Table 2 shows the effect of a number of inhibitors on the rate of enzymic reduction of heart-muscle cytochrome *c* in the presence of 0·1 mM potassium cyanide. A slow rate of reduction of cytochrome *c* which occurred in the absence of substrate was insensitive to inhibitors.

With L-malate, the activity was markedly stimulated by addition of DPN, but there was no effect on activity with succinate.

Concentrations of inhibitors greater than those shown in Table 2 caused no further inhibition of the rates with DPNH or with succinate as substrates. Sodium malonate, in equimolar concentration with succinate (0·05M), caused almost complete inhibition of succinate–cytochrome *c* reductase activity.

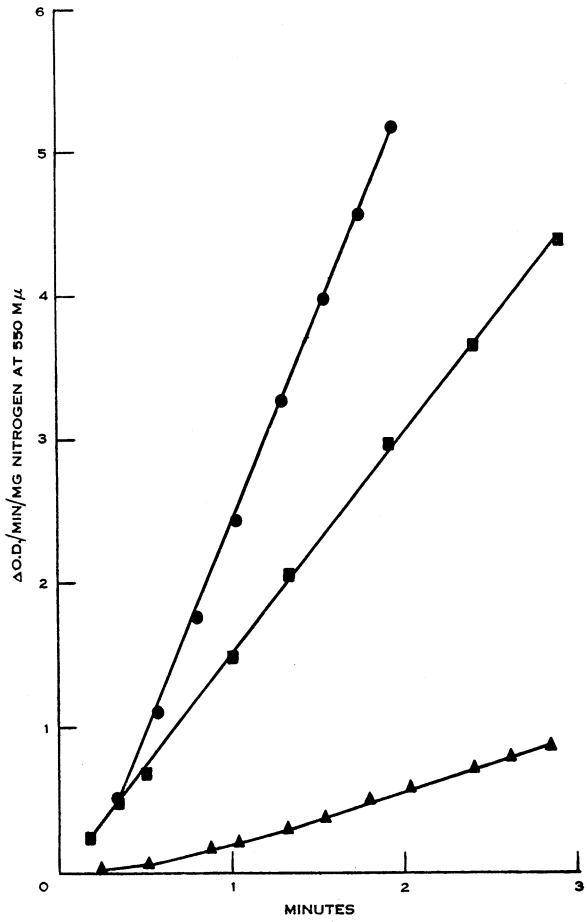


Fig. 1.—Effect of freezing and of hypotonic treatment on the succinate–cytochrome *c* reductase activity of beetroot mitochondria. Activities were determined at pH 7·2 and at 25°C under the conditions given in Table 2. ▲ Freshly isolated mitochondria; ■ after storage in 0·4M sucrose at −15°C for 3 days; ● after treatment with water for 30 min.

With succinate as substrate, there was consistently an initial lag of 1–2 min before a linear rate of reduction of cytochrome *c* was obtained. As shown in Figure 1, this lag was eliminated when the mitochondria were damaged structurally by freezing and thawing or by exposure to hypotonic solutions. These treatments also caused a marked stimulation of the activity.

(*d*) *Dichlorophenolindophenol* (*Dye*) *Reductase Activity*

Dye reduction was measured anaerobically with 0·1 mM potassium cyanide (Table 3). With succinate, the activity was low but was stimulated by addition of 5 mM EDTA (control, 31; with EDTA, 43 O.D. units/hr/mg nitrogen), possibly due to

TABLE 2

EFFECT OF SOME INHIBITORS ON THE CYTOCHROME *c* REDUCTASE ACTIVITY OF BEETROOT MITOCHONDRIA WITH VARIOUS SUBSTRATES

Reaction mixtures, at pH 7·2, contained 0·27M sucrose, 31 mM TRIS, 28 mM acetic acid, 0·1 mM KCN, 60 μM heart-muscle cytochrome *c*, and 59 μg mitochondrial nitrogen per reaction mixture (3 ml), and with 0·26 mM DPNH or 50 mM sodium succinate or malate as substrates

Substrate	DPN Concn. (mM)	Control Rate (Δ O.D./hr/mg N at 550 mμ)	Percentage Inhibition with:			
			Antimycin A (44 μg)	Phenyl-urethane (2·7 mM)	Ethanol (2·7%, v/v)	Amytal (3·6 mM)
DPNH	0	192	49	28	16	43
Succinate	0	106	88	55	0	0
Malate	0	31	80	65	—	74
Malate	0·07	76	70	57	—	49
Malate	0·13	86	55	52	—	44

chelation of toxic material in the dye preparation. There was no effect of EDTA on the activity with DPNH. A non-enzymic reduction of dye by DPNH at about 5 per cent. of the enzymic rate was observed.

TABLE 3

EFFECT OF INHIBITORS ON THE ENZYMIC REDUCTION OF 2,6-DICHLORO-PHENOLINDOPHENOL BY BEETROOT MITOCHONDRIA

Reaction mixture, at pH 7·2, contained 0·27M sucrose, 31 mM TRIS, 28 mM acetic acid, 0·1 mM KCN, 5 mM EDTA, 0·04 per cent. 2,6-dichloro-phenolindophenol, either 0·26 mM DPNH or 50 mM sodium succinate, and 53 μg of mitochondrial nitrogen per reaction mixture (3 ml)

Substrate	Control Rate (Δ O.D./hr/mg N at 600 mμ)	Percentage Inhibition with:	
		Amytal (3·6 mM)	Malonate (0·03M)
DPNH	74	25	0
Succinate	43	0	69

At similar concentrations to those shown in Table 2, there was no inhibition by antimycin A, phenylurethane, or ethanol with either DPNH or succinate as

substrates. However, as shown in Table 3, amytal inhibited activity with DPNH, and malonate inhibited activity with succinate as substrate.

(*e*) *Effects of Storage and other Treatments on Enzymic Activities*

There was no decline in oxidase or reductase activities when freshly isolated suspensions of the mitochondria were kept in an ice-bath for 1–1½ hr. Freezing and thawing of suspensions stimulated all enzymic activities investigated. The increased activities were subsequently maintained by storage of the mitochondria in 0·4M sucrose at −15°C for several days, but declined after 17 days (Table 4).

TABLE 4

EFFECT OF STORAGE AT −15°C ON THE ENZYMIC PROPERTIES OF BEETROOT MITOCHONDRIA IN 0·4M SUCROSE

Reaction mixture, at pH 7·2, contained 0·27M sucrose, 31 mM TRIS, 28 mM acetic acid, 0·26 mM DPNH or 50 mM sodium succinate where indicated, and 43 μg of mitochondrial nitrogen per reaction mixture (3 ml). The following additions were made for the enzymic activities indicated: DPNH oxidase, 2 μM heart-muscle ferricytochrome *c*; cytochrome *c* reductase, 60 μM heart-muscle ferricytochrome *c* and 0·1 mM KCN; cytochrome *c* oxidase, 60 μM ferrocytochrome *c*; dichlorophenolindophenol (dye) reductase, 0·04 per cent. 2,6-dichlorophenolindophenol, 5 mM EDTA, and 0·1 mM KCN

Enzymic System	Activity (Δ O.D./hr/mg N)		
	On Isolation	3 Days Storage	17 Days Storage
DPNH oxidase*	53	101	87
DPNH–cytochrome *c* reductase†	192	275	241
Succinate–cytochrome *c* reductase†	104	(85)§	170
Cytochrome *c* oxidase†	122	133	90
DPNH–dye reductase‡	154	328	114
Succinate–dye reductase‡	34	65	58

* Measured at 340 mμ.

† Measured at 550 mμ.

‡ Measured at 600 mμ.

§ This result is atypical; usually an increase of activity as compared with the control was obtained, as in Table 5.

A suspension of fresh mitochondria in 0·4M sucrose was centrifuged and the sedimented mitochondria were resuspended in water for 5 min at 0°C and then sucrose was added and the suspension made up to the original volume in 0·4M sucrose. As shown in Table 5, this hypotonic treatment caused a marked stimulation of the enzymic activities, comparable with that obtained by freezing and thawing.

(*f*) *Oxidative Phosphorylation*

As shown by Table 6, the mitochondria catalyse esterification of inorganic phosphate coupled to oxidation of succinate or malate. Addition of DPN stimulated

oxygen uptake with malate but depressed the P/O ratio. Storage of the mitochondria in 0·4M sucrose at −15°C for 7 days caused a decline in oxygen uptake and in the P/O ratios with malate alone, and with malate and DPN.

TABLE 5

COMPARISON OF FREEZING AND HYPOTONIC TREATMENTS ON THE ENZYMIC ACTIVITIES OF BEETROOT MITOCHONDRIA

Reaction mixture, at pH 7·2, contained 0·27M sucrose, 31 mM TRIS, 28 mM acetic acid, 0·26 mM DPNH or 50 mM sodium succinate where indicated, and 43 μg of mitochondrial nitrogen per reaction mixture (3 ml). The following additions were made for the enzymic activities indicated: DPNH oxidase, 2 μM heart-muscle ferricytochrome *c*; cytochrome *c* reductase, 60 μM heart-muscle ferricytochrome *c* and 0·1 mM KCN; cytochrome *c* oxidase, 60 μM ferrocytochrome *c*; dichlorophenolindophenol (dye) reductase, 0·04 per cent. 2,6-dichlorophenolindophenol, 5 mM EDTA, and 0·1 mM KCN

Enzymic System	Activity (Δ O.D./hr/mg N)		
	Control	After Freezing Treatment	After Hypotonic Treatment
DPNH oxidase	52	110	119
DPNH–cytochrome *c* reductase	150	259	290
Succinate–cytochrome *c* reductase	36	150	174
Cytochrome *c* oxidase	108	330	570
DPNH–dye reductase	154	224	254

TABLE 6

ESTERIFICATION OF INORGANIC PHOSPHATE COUPLED WITH OXIDATION OF SUCCINATE AND MALATE BY BEETROOT MITOCHONDRIA

Reaction mixture (3 ml) contained 0·27M sucrose, 31 mM TRIS, 28 mM acetic acid, 2 μM heart-muscle cytochrome *c*, 13 mM K_2HPO_4, 1 mM ADP, 6·7 mM $MgCl_2$, 10 mM NaF, 20 mM glucose, 50 mM sodium succinate or malate, and excess hexokinase. Oxygen uptake was determined manometrically at 25°C

Substrate	Oxygen Uptake (μg-atoms)	ATP Formed† (μmoles)	P/O Ratio
Succinate	6·7	5·2	0·78
Malate	6·7	13·4	2·00
Malate + 0·48M DPN	9·9	13·9	1·40
Malate*	2·2	3·7	1·68
Malate + 0·48 mM DPN*	5·1	6·1	1·20

* Mitochondrial preparation stored for 7 days at −15°C.

† Equivalent to μg-atoms of inorganic phosphate esterified; ATP estimated as μmoles of glucose 6-phosphate formed.

Figure 2 shows that with freshly isolated mitochondria, the rate of oxygen uptake with succinate alone was linear for about 30 min but then declined. How-

ever, under the conditions necessary for oxidative phosphorylation, the oxygen uptake was linear for at least 80 min.

(*g*) *Cytochrome Pigments*

Direct spectroscopic observations of pellets of sedimented mitochondria and suspensions in 0·4M sucrose were made with a low-dispersion spectroscope. Materials were observed at about 0°C and after cooling to about −190°C in liquid air. With pellets and heavy suspensions of particles it has been found unnecessary to use 50 per cent. glycerol for intensification of the absorption bands at −190°C (cf. Keilin and Hartree 1949).

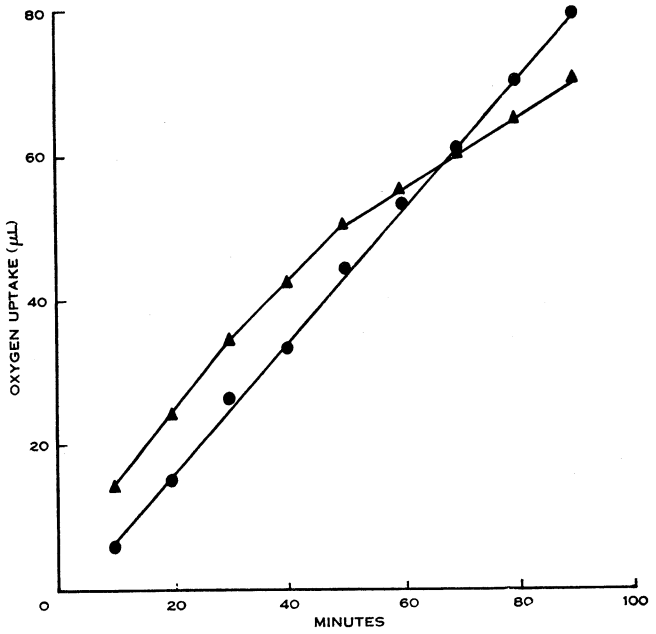


Fig. 2.—Oxidation of succinate by beetroot mitochondria. Activities were determined manometrically (at 25°C) with air as the gas phase. The reaction mixture (3 ml, pH 7·2) contained 0·27M sucrose, 31 mM TRIS, 28 mM acetic acid, 2 μM heart-muscle cytochrome *c*, and 50 mM sodium succinate and 370 μg of mitochondrial nitrogen. ▲ Control (no additions); ● with 1 mM ADP, 6·7 mM $MgCl_2$, 10 mM NaF, 20 mM glucose, 13 mM potassium phosphate, and excess hexokinase.

When freshly isolated, beetroot mitochondria are chocolate-brown in colour, and show no distinct absorption bands of haemoprotein pigments which are probably oxidized. On occasions, a weak broad band between 535 and 550 mμ is seen. The mitochondria also show a strong, broad band near 490 mμ which, however, does not change with oxidation and reduction and is probably not due to a haemoprotein. This component occurs in mitochondria from silver-beet petioles and from wheat roots (Martin and Morton 1957).

When reduced with succinate anaerobically at 0°C, the mitochondria were pale brown and showed asymmetric absorption areas between 518 and 530 mμ, between

550 and 562 mμ, and at about 595–598 mμ. These bands were much intensified by addition of sodium dithionite. The broad band between 550 and 562 mμ was resolved into three bands, attributed to the α-absorption bands of cytochrome *b* (558–562 mμ), cytochrome c_1 (552–555 mμ), and cytochrome *c* (550 mμ). The resolution of these bands is assisted by examination after treatment with liquid air (−190°C). However, this is also accompanied by a slight shift of the band positions towards lower wavelengths as well as a "splitting" of certain bands, so that identification of components is more complicated than by examination at 0°C.

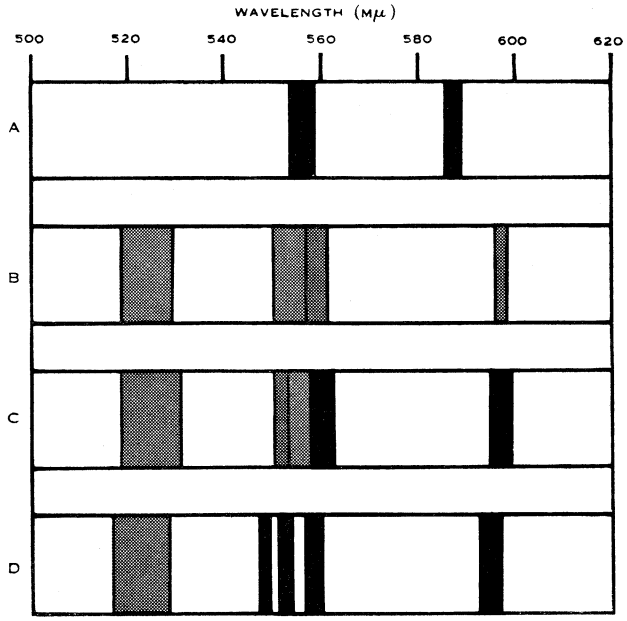


Fig. 3.—Diagrammatic representation of visible absorption bands of beet mitochondrial haem pigments: *A*, with alkaline pyridine and sodium dithionite at 0°C; *B*, with sodium succinate and anaerobic conditions at 0°C; *C*, with sodium dithionite at 0°C; *D*, with sodium dithionite at −190°C.

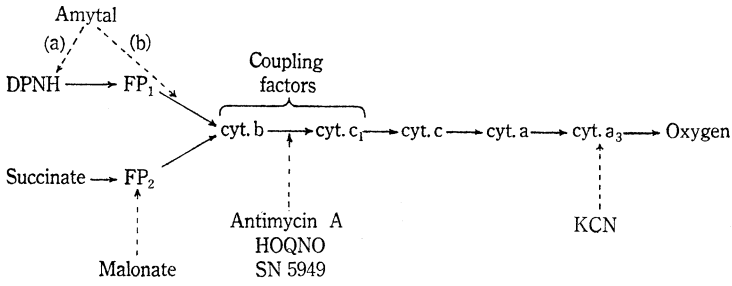
After treatment of the mitochondria with alkaline pyridine and sodium dithionite, intense α- and β-absorption bands of the pyridine haemochromes of protohaem (at 557 mμ) and of haem *a* (at 588 mμ) were observed, as well as a very weak α-band of the haemochromogen of haem *c* (near 550 mμ). Figure 3 shows the relative positions and relative intensities of the absorption bands observed.

IV. Discussion

The beetroot mitochondria used in this work appeared to be substantially free of contamination by plastids and microsomes, when electron micrographs of preparations fixed with osmium tetroxide were examined. The observations therefore relate only to mitochondria.

The spectroscopically detectable haemochromes have been identified as cytochromes ($a+a_3$), b, c_1, and c (Fig. 3), similar to the cytochromes of mitochondria from beet petioles and wheat roots (Martin and Morton 1957). However, these components are not necessarily identical with the analogous cytochromes of mitochondria from animal tissues. Lundegårdh (1952, 1958) and Martin and Morton (1957) identified cytochrome a_3 in plant tissues and this finding is supported by numerous reports of the complete inhibition of respiratory activity by potassium cyanide (0·1 mM) as found in this work. The absorption band at 595–598 mμ (Fig. 3) is therefore attributed to cytochrome ($a+a_3$) and this is regarded as the terminal component of the respiratory chain. As compared with wheat-root mitochondria, beetroot mitochondria have a relatively large amount of cytochrome a-type component and relatively small amount of cytochrome c. This is consistent with the observed stimulation of DPNH oxidase activity by added cytochrome c (Table 1).

The component with an α-band at about 554 mμ (Fig. 3) is similar to that observed in other plant mitochondria and called by Martin and Morton (1957) "cytochrome c_1". Like cytochrome b (α-band 558–562 mμ) it is believed to be a component of the respiratory chain, since it is reduced by DPNH and succinate. A similar component has been observed by other workers (Crane 1957; Hackett and Haas 1958; and Lundegårdh 1958). Until this cytochrome is isolated, and the nature of the prosthetic group is identified, the name "cytochrome c_1" must be regarded as tentative and not as implying identity with cytochrome c_1 of animal tissues (see Morton 1958). From these observations, the respiratory chain* of the beetroot mitochondria is assumed to comprise the following components at least:



Here, FP_1 and FP_2 represent different flavoproteins.

The probable sites of action of inhibitors are indicated. Whereas Ernster *et al.* (1955) consider amytal to act between the flavoprotein (FP_1) and cytochrome b (site (b)), Chance and Williams (1956) consider it to act between DPNH and flavoprotein (site (a)). The site of action of antimycin A was identified by Potter and Reif (1952) for animal tissue and by Martin and Morton (1957) for plant mitochondria. HOQNO (Lightbown and Jackson 1956; Jackson and Lightbown 1958) and SN 5949 (Ball, Anfinsen, and Cooper 1947) each appear to act at a site similar to that at which antimycin A acts in animal tissues. Chance and Hackett (1959) have recently described the effects of HOQNO and some other inhibitors on the

* Lester and Crane (1959) have reported substantial amounts of naphthoquinones related to ubiquinone (R. A. Morton 1958) in plant mitochondria; whether ubiquinone is a component of the respiratory chain is unknown.

respiratory chain of mitochondria of skunk cabbage (*Sympflocarpus foetidus*). Chance and Sacktor (1958) and Chance (1958) have observed a substantial increase in the apparent amount of cytochrome *b* of animal mitochondria when inhibited with antimycin A, or reduced with dithionite, as compared with substrate reduction anaerobically. This behaviour is particularly apparent with plant mitochondria (Martin and Morton, unpublished data). Antimycin A may combine with cytochrome *b* itself.

The activities of individual enzymic steps of the respiratory chain have been compared by assuming that the oxidation of DPNH involves two hydrogen equivalents and the reduction of cytochrome *c* only one, and by expressing all rates as μmoles of oxygen uptake/hr/mg mitochondrial nitrogen, as shown in Table 7.

TABLE 7

COMPARISON OF THE OXIDATION CAPACITY OF VARIOUS ENZYME SYSTEMS OF BEETROOT MITOCHONDRIA

Assay details for each of the systems are given in the text and in Tables 1–6. The mean and range of values (shown in parenthesis) are for determinations on at least five different preparations

System	Wavelength for Estimation (mμ)	Δ O.D./hr/mg N	Oxygen Consumed (μl/hr/mg N)
DPNH oxidase	340	63	340 (286–378)
DPNH–cytochrome *c* reductase	550	155	133 (105–165)
Succinate–cytochrome *c* reductase	550	87	75 (48– 96)
Succinate oxidase	—	—	472 (314–637)
Cytochrome *c* oxidase	550	116	99 (84–116)
DPNH–dye reductase	600	157	276 (188–342)
Succinate dehydrogenase	600	37	65 (56–144)
Malate oxidase	—	—	233 (206–301)
Malate oxidase*	—	—	323 (257–445)

* With 0·48 mM DPN (see Table 6).

Under the conditions of the experiments, the rate of oxidation of all substrates is a maximum when a small concentration of cytochrome *c* is added and oxygen is the terminal acceptor. Moreover, the maximum inhibition of DPNH oxidation by antimycin A, phenylurethane, and amytal occurs under these conditions (Table 1). High concentrations of added cytochrome *c* interfere with the normal sequence of reactions as indicated by the decreased inhibition of DPNH–cytochrome *c* reductase (Table 2) when compared with DPNH oxidase activity (Table 1), as well as by the lower rate of substrate oxidation with cytochrome *c* as the terminal acceptor (Table 7). The lower inhibition of the cytochrome *c* reductase activities (Table 2) is not due to binding of the inhibitors by added cytochrome *c*, since Reif and Potter (1953) have shown that cytochrome *c* does not bind antimycin A. Moreover, increasing the concentrations of inhibitors did not increase the percentage inhibition, and succinate–cytochrome *c* reductase activity was 88 per cent. inhibited by the antimycin A (Table 2).

It is possible that the mitochondria had lost some cytochrome *c* by extraction during isolation. This would account for the very small amount of cytochrome *c* detected spectroscopically, and for the marked influence of added cytochrome *c* on the enzymic activities. Mitochondria prepared by Martin and Morton (1957) from silver-beet petioles and from wheat roots also had relatively small amounts of cytochrome *c*.

In beetroot mitochondria, as in mitochondria from other plants (Martin and Morton 1956*a*, 1956*b*), there appear to be at least two pathways for oxidation of DPNH, one of which is sensitive, the other insensitive, to antimycin A. The pathway insensitive to antimycin A is stimulated by addition of large amounts of heart-muscle cytochrome *c* or of DPNH, and it is possible that it represents an "external" or non-phosphorylating system for oxidation of DPNH in solution as distinct from "bound" DPNH of mitochondria (cf. Lehninger 1951, Ernster *et al.* 1955).

The inhibitory effect of ethanol observed in these experiments was not specific for any one step in the electron transport chain. Beer and Quastel (1958) reported that dilute ethanol partially inhibited the salt-stimulated respiration of brain cortex slices but had no effect on the respiration of the isolated mitochondria. Keilin and Hartree (1940) found that a high concentration of ethanol (45 per cent.) irreversibly modified the cytochromes a_3, *a*, and *b* and thus completely inhibited their succinate–cytochrome *c* reductase system. A similar effect could have occurred to the beet mitochondrial system studied here, but to such a small degree that it was noticeable only with the efficient DPNH oxidation process.

The stimulation of all enzymic systems of beetroot mitochondria by freezing and thawing and by exposure to hypotonic conditions (Table 4 and 5) is probably due to the improved permeability to substrates. Whereas with freshly isolated mitochondria there is a pronounced lag in the reduction of cytochrome *c* by succinate, this is not observed in the modified mitochondria (Fig. 1). The stability of the enzymic systems of beetroot mitochondria including those systems involved in oxidative phosphorylation is particularly noteworthy. Whereas Martin and Morton (1956*b*) observed that the succinate dehydrogenase of mitochondria from wheat root rapidly lost activity on storage, the beetroot mitochondria retain activity when held at −15°C for some days. These differences probably reflect small differences in the structure of the mitochondria from the two sources and emphasize the caution necessary in extending results obtained with one plant tissue to another tissue of the same plant, or to a similar tissue of a different species.

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