5. A Fe^{2+} ion-ethylenediaminetetra-acetic acid complex can catalyse the oxidative formation of an inhibitor from schradan.

6. A mechanism for the simultaneous oxidation of schradan and reduced triphosphopyridine nucleotide is suggested.

7. The requirements for the conversion of dimefox to an anti-esterase are the same as those for schradan, but the rate of esterase inhibition is much higher. Dimefox is also considerably more toxic than schradan to the locust, but LD_{50} does not vary with fat-body converting activity.

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Cytochromes and some Respiratory Enzymes in Mitochondria from the Spadix of *Arum maculatum*

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The discovery by James & Beevers (1950) that the rapid respiration of the *Arum* spadix is unaffected by cyanide stimulated interest in the mitochondria of this tissue. Mitochondria isolated from the spadix of *Arum maculatum* have been shown to be similar to those obtained from many other plant and animal tissues in that they are able to oxidize intermediates of the citric acid cycle (Hackett & Simon, 1954; James & Elliott, 1955) and contain cytochromes *a*, *b* and *c* (Bendall & Hill, 1956). However, these oxidations are inhibited to only a small extent by cyanide (James & Elliott, 1955) and are exceptionally rapid. It has also been shown (Bendall & Hill, 1956) that in addition to the normal cytochrome system *Arum* mitochondria contain a relatively large amount of cytochrome *b*, which can be oxidized by air in the presence of

cyanide. It was suggested that cytochrome *b*, accounts for a significant part of the cyanide-stable respiration of the mitochondria.

Simon (1957) has recently measured the rates of oxidation of succinate and cytochrome *c* at different stages of development of the spadix, and determined the effect of cyanide and antimycin A on these enzyme systems. He has shown that the succinic-oxidation system rapidly increases in activity as the spadix grows and matures, and that in the mature spadix cytochrome *c* oxidase and the succinic system are about equally active, despite the comparatively small effect of cyanide on the succinic system.

Further studies on the respiratory enzymes and cytochromes in *Arum* mitochondria are described in the present paper. The effects of cyanide and

antimycin A were investigated in some detail, and the properties of mitochondria from young and mature spadices have been compared.

MATERIALS AND METHODS

Plant material. Inflorescences of *Arum maculatum* were collected from wild colonies near Cambridge either when still growing, when the spathe was approx. 8 cm. tall and only just emerging, or just before the opening of the spathe. They were either used immediately or stored at 4°. The experimental material was the sterile head of the spadix, after removal of the papillae and coloured cell contents of the epidermis in the mature spadices. Young spadices which had already become pigmented were rejected.

On the suggestion of Dr C. T. Prime corms of *A. neglectum* (Towns.) Ridley (*A. italicum* var. *neglectum* Towns.; *A. italicum* auct. angl. non Mill.) were collected in August 1954 from a colony at Arundel. The plants were grown in the University Botanic Gardens by courtesy of Mr J. S. L. Gilmour. The inflorescences were harvested just before the opening of the spathe and stored as above.

Peas (*Pisum sativum* var. *Gradus*) were purchased locally. They were washed under a running tap for half an hour, and soaked in water for 6 hr. and germinated in moist vermiculite for 3½ days in the dark at room temperature (about 25°).

Preparation of mitochondrial fractions. The method of preparation of *Arum* mitochondrial fractions was the same as that described in a previous paper (Bendall & Hill, 1956). The washed mitochondria were suspended in 0.20M-sucrose containing 0.05M-phosphate (KH_2PO_4 - Na_2HPO_4), pH 7.1, for spectroscopic experiments. For manometric experiments they were suspended in 0.25M-sucrose before addition to the manometer vessels.

In the preparation of mitochondrial fractions from pea seedlings the cotyledons only were used. Tissue (30 g.) was ground with 40 ml. of 0.4M-sucrose containing 0.1M-phosphate (KH_2PO_4 - Na_2HPO_4), pH 7.1. The brei was strained through muslin and centrifuged for 5 min. at 1700 g in an International refrigerated centrifuge, model PR-1, which precipitated mainly starch grains. The supernatant was centrifuged for 15 min. at 14 000 g to give the mitochondrial fraction. The mitochondria were washed once by suspending the pellet in the grinding medium and recentrifuging. The washed mitochondria were then suspended in a small volume of grinding medium.

Rates of oxygen uptake by mitochondria. Uptake of O_2 was followed for 1 hr. in Warburg manometers at 20°. The rates were fairly steady, but usually fell off a little during the second half hour; initial rates only are quoted. Carbon dioxide was absorbed with KOH and filter paper in the centre well, or with the $\text{Ca}(\text{CN})_2$ - $\text{Ca}(\text{OH})_2$ mixtures described by Robbie (1948) when cyanide was present in the reaction mixture.

The reactions were started by adding the mitochondrial suspension to the flasks before attaching them to the manometers. Cyanide and antimycin A were added after the mitochondria.

Convenient rates of uptake of O_2 were obtained for measurement of rates of succinate oxidation when mitochondria prepared from 0.1 g. of mature spadix or 1 g. of

young spadix were used in each manometer flask. These weights are rough indications only, and the actual quantity of mitochondria used varied somewhat from one experiment to another. A quantitative recovery of mitochondria was not attempted. The endogenous uptake of O_2 of washed mitochondria was less than 30 $\mu\text{l.}$ of O_2 /mg. of N/hr. for mature spadices, and less than 10 $\mu\text{l.}$ of O_2 /mg. of N/hr. for young spadices.

Oxygen uptake of tissue slices. A large number of slices were cut about 0.2 mm. thick with the aid of a hand microtome, and suspended in 0.05M-phosphate (KH_2PO_4 - Na_2HPO_4), pH 7.1, and washed in the same buffer. Slices (50) were transferred to each manometer flask, which contained 3 ml. of 0.05M-phosphate, pH 7.1, or 3 ml. of buffer containing mM-HCN in the main compartment, and 20% (w/v) KOH or the appropriate $\text{Ca}(\text{CN})_2$ - $\text{Ca}(\text{OH})_2$ mixture in the centre well. The temperature was 30°.

Succinic-cytochrome c reductase activity. This was measured spectrophotometrically by following the reduction of cytochrome c at 550 m μ in a Beckman spectrophotometer model DU at room temperature (about 20°). A value of 18.5 was used for the difference between ϵ_{mm} of the reduced and oxidized forms (Margoliash, 1954). HCN (mm) was added to the reaction mixture to inhibit cytochrome c oxidase. In a few cases HCN was omitted and the reaction was carried out in an evacuated Thunberg tube specially made for use in a spectrophotometer (obtainable from the Thermal Syndicate Ltd., Wallsend, Northumberland). Small differences in rate were observed between the anaerobic and cyanide-treated particles, but the results were not consistent and HCN was normally added for convenience. The optical density of the experimental cell was measured against a blank containing mitochondria and all reagents except cytochrome c.

Spectroscopic observations. These were made with a Zeiss microspectroscope ocular. The scale was set with the α -band of reduced cytochrome c at 550 m μ . For observations at room temperature the mitochondrial suspensions, about 5–10 g. of fresh tissue/ml., were held in a 1 cm. glass cell with optical faces. For observation of spectra at the temperature of liquid N_2 the mitochondrial suspension was mixed with an equal volume of glycerol and examined as described by Keilin & Hartree (1949).

Cytochromes were usually reduced by the addition either of one drop of M-sodium succinate, pH 7.1, or of a few crystals of sodium dithionite to 1.5–2.0 ml. of mitochondrial suspension.

Cytochrome concentrations. The intensities of the α -bands of cytochrome were measured by comparison with suitable standards in the spectrophotometer of Hill (1936).

For cytochrome c the standard was a solution of purified heart-muscle cytochrome c which was estimated by determination of the extinction at 550 m μ with a Beckman spectrophotometer after addition of $\text{Na}_2\text{S}_2\text{O}_4$. A molar-extinction coefficient of $2.7 \times 10^3 \text{ l. moles}^{-1} \text{ cm}^2$ was used (Margoliash, 1954).

Cytochrome b was compared with the α -band of cytochrome b in a suspension of baker's yeast in saturated sucrose solution in the colorimeter cup. It was judged that the intensity of the b band in the yeast suspension was 70% of that of the c band. It was then assumed that the millimolar extinction coefficients of yeast cytochrome b and *Arum* cytochromes b and b_7 are equal and have a value of 33, which is the value for pyridine haemochromogen

(Lemberg & Legge, 1949) and crystalline yeast cytochrome b_2 (Appelby & Morton, 1954).

At the suggestion of Dr R. Hill cytochromes $a + a_3$ were estimated by comparison with the band at 605 $m\mu$ of a dilute solution of a chlorinated resorufin derivative, which was probably the tetrachloro compound. The dye was prepared by Dr Hill by the following method. Resorufin (10 mg.) was suspended in 5 ml. of water and dissolved by the addition of 0.5 ml. of 0.1 N-NaOH. An aqueous solution (100 ml.) containing 1% (w/v) of NaHCO_3 and 1% (w/v) of KCl was added. The mixture was treated at room temperature during 20 min. with excess of sodium hypochlorite solution (0.4 ml., 10–14% of available chlorine). The solution should be between pH 8 and 8.55. The sparingly soluble potassium salt of chlororesorufin was centrifuged off and washed twice with the NaHCO_3 -KCl mixture. It was reprecipitated by dissolving in 50 ml. of boiling water, followed by the addition of 0.5 g. of KCl and cooling. For use as a standard the potassium salt was dissolved in 90% acetone containing a trace of NaHCO_3 , and about an equal volume of methanol was added to bring the position of the main absorption band to the desired value in the region of 605 $m\mu$. The extinction at the peak of the band was determined spectrophotometrically, and the value of 20 given by Dannenberg & Kiese (1952) for the millimolar extinction coefficient of heart-muscle cytochrome a at 606 $m\mu$ was used.

The above method of estimating cytochromes $a + a_3$ was not available in earlier experiments. In these, yeast suspensions were used as standards and their cytochrome c contents were estimated by comparison with purified cytochrome c . It was assumed that the ratio of $c:a$ is approximately constant in different samples of yeast and has the value 1.1, which is the mean of two values (0.99 and 1.21) obtained by comparison with cytochrome c and chlororesorufin.

The maxima of absorption bands were matched. The methods for the estimation of cytochromes c and $b + b_7$ do not allow for the contributions from minor components with absorption bands at 563 and 553 $m\mu$ at -196° (see Results).

The absolute cytochrome concentrations expressed in terms of mitochondrial nitrogen are only approximate because multiple reflexions in the turbid suspensions may cause an error in the estimation of the light path (Holton, 1955).

Nitrogen. The nitrogen content of mitochondrial suspensions was determined by micro-Kjeldahl digestion as described by Chibnall, Rees & Williams (1943), followed by distillation and titration.

Dry weights. Dry weights of tissue slices and whole-spadix appendages were determined after drying in an oven at 105° .

Reagents. Barium adenosine triphosphate was prepared from muscle by the late Mr E. J. Morgan by a method based on that of Needham (1942). Solutions of sodium adenosine triphosphate (0.05 M) were prepared by the method of Bailey (1942) and stored at -20° . In some experiments disodium adenosine triphosphate supplied by Pabst Laboratories, Milwaukee, Wis., U.S.A., was used.

Cytochrome c was prepared by Mr D. C. Gardiner by the method of Keilin & Hartree (1945) up to the 0.34% Fe stage.

A concentrated solution of A.R. succinic acid was

neutralized with NaOH, treated with 8-hydroxyquinoline to remove heavy metals (Chappell & Perry, 1953), and the disodium salt was precipitated with ethanol. α -Oxoglutaric acid, which was prepared by Mr B. R. Slater, contained approx. 0.5% of succinic acid, as measured with a Keilin & Hartree (1947) heart-muscle preparation. L-Malic acid was supplied by L. Light and Co. Ltd.

Crystalline antimycin A was supplied by the Wisconsin Alumni Research Foundation. Stock solutions (100 and 500 $\mu\text{g./ml.}$) in 96% ethanol were stored at 4° .

RESULTS

Cytochrome components

The cytochrome spectrum of mitochondria from the mature spadix has already been described (Bendall & Hill, 1956). In the presence of sodium dithionite the α -bands of cytochromes a and c can be seen, and also a strong band at 560 $m\mu$ which is due mainly to cytochrome b_7 (Fig. 1a). When a concentrated suspension of mitochondria from young spadices (10 g. of fresh tissue/ml.) was reduced with dithionite and examined with a microspectroscope the α -bands of cytochromes a and c were also visible, but the b band at 561 $m\mu$ was relatively weak compared with the corresponding band in the mature spadices, being now only slightly stronger than the c band (Fig. 1c).

This spectrum therefore more closely resembles the spectrum of mitochondria from germinating-pea cotyledons (Fig. 1e) than that of the mature *Arum* mitochondria. When mitochondria from the young spadix or from pea cotyledons were reduced with succinate instead of dithionite the b band was sharper and slightly less intense. The spectra were then similar to the spectrum of baker's yeast. A weak chlorophyll band was often visible at about 670 $m\mu$ in preparations from young *Arum* spadices.

In the presence of approx. mM-cyanide and sodium succinate (approx. 0.03 M) it was found possible to obtain partial oxidation of the b components of the young *Arum* mitochondria by shaking at 0° with air. This suggested that the young mitochondria also contain some cytochrome b_7 .

The concentrations of cytochromes in young- and mature-spadix mitochondria are shown in Table 1. The scatter of results for the absolute concentrations in the young mitochondria do not allow any definite conclusions to be drawn concerning the changes of concentration as the spadix matures. However, the relative concentrations are more consistent and it is clear that the concentration of b components relative to that of cytochrome c increases about three times.

When a suspension of mature *Arum* mitochondria was reduced with sodium dithionite, and cooled in liquid nitrogen and rapidly examined with a microspectroscope, the absorption bands showed the

expected sharpening and were now located at 600, 558 and 549 $m\mu$, with faint sharp bands also at 536 and 528 $m\mu$ (Fig. 1*b*). A faint satellite band of cytochrome *c* at about 546 $m\mu$ (Keilin & Hartree, 1949) could sometimes be seen, but it was not as distinct as in purified heart-muscle cytochrome *c* (see Estabrook, 1956). A faint sharp band could occasionally be seen at 563 $m\mu$. When the *b* components were only partially reduced with succinate this band was more distinct, and at the same time another band appeared at 554 $m\mu$, which may be due to cytochrome *c*₁.

The spectrum of young-spadix mitochondria reduced with sodium dithionite and cooled in liquid nitrogen was similar to the spectrum of the partially oxidized mature mitochondria (Fig. 1*d*). Pea-cotyledon mitochondria reduced with dithionite and examined in the same manner showed similar bands with slightly different relative intensities at 564, 558, 554 and 549–550 $m\mu$ (Fig. 1*f*).

The α -band of the reduced cytochrome *a*₃-cyanide complex was observed in *Arum* mitochondria in the following manner. A concentrated suspension of mature mitochondria was placed in a Thunberg tube, a little solid sodium succinate was added to the suspension, and a few milligrams of solid potassium cyanide were placed in the stopper. The tube was thoroughly evacuated and the cyanide tipped in. A shading extending to 590 $m\mu$ was now observed on the short-wavelength side of the α -band of cytochrome *a*. The shading was approximately half as intense as the band at 603 $m\mu$, so the ratio *a*₃:*a* is probably smaller than in heart muscle. On admitting air to the tube the shading disappeared and did not reappear on addition of dithionite.

The spectrum of mitochondria from the spadix of *A. neglectum* was intermediate between the spectra of the mitochondria from young and mature spadices of *A. maculatum*. The *b* cytochrome components of mitochondria from *A. neglectum* could be completely oxidized in the presence of succinate and cyanide by shaking at 0° with air.

Fig. 1. Diagram showing the cytochrome spectra of *Arum* and pea-cotyledon mitochondria. (a) Mitochondria from mature spadices of *A. maculatum* + $\text{Na}_2\text{S}_2\text{O}_4$ at room temperature (20°). (b) The same at -196° . (c) Mitochondria from young spadices of *A. maculatum* + $\text{Na}_2\text{S}_2\text{O}_4$ at room temperature. (d) The same at -196° . (e) Mitochondria from cotyledons of germinating peas + $\text{Na}_2\text{S}_2\text{O}_4$ at room temperature. (f) The same at -196° . (g) Mitochondria from mature spadices of *A. maculatum* + succinate and antimycin A and shaken with air at room temperature. (h) The same after cooling to -196° . Effects of carotenoids and light scattering on the blue end of the spectrum are not shown. The band at 670 $m\mu$ in (c) and (d) is due to chlorophyll. Intensities of absorption bands may be compared within a particular spectrum, but not between spectra.

Table 1. *Cytochrome concentrations*

Results are expressed as μ moles of haem/g. of mitochondrial N. Cytochrome (*a* + *a*₃) concentrations shown in bold type were obtained indirectly by comparison with yeast (see Materials and Methods).

Date of experiment	Age of spadix	<i>a</i> + <i>a</i> ₃	<i>b</i> + <i>b</i> ₇	<i>c</i>	Ratio (<i>b</i> + <i>b</i> ₇)/ <i>c</i>
18 April 1955	Young	3.6	3.7	2.0	1.8
18 April 1956	Young	0.97	1.3	0.84	1.6
27 April 1956	Young	1.6	2.0	1.5	1.3
20 May 1955	Mature	2.0	3.6	0.74	4.9
2 June 1955*	Mature	2.1	4.2	0.79	5.3
7 June 1955*	Mature	1.2	2.1	0.64	3.3

* Inflorescences were collected 30 May 1955.

Table 2. *Effect of cyanide on the oxidation of citric acid-cycle intermediates*

Control rates of oxygen uptake are expressed as $\mu\text{l. of O}_2/\text{mg. of mitochondrial N/hr.}$ (1 mg. of mitochondrial N corresponded to 2.56 g. of fresh young spadix and 1.30 g. of mature spadix). Each reaction vessel contained 0.2M-sucrose, 0.05M-phosphate ($\text{KH}_2\text{PO}_4\text{--Na}_2\text{HPO}_4$), pH 7.1, 0.33 mM-adenosine triphosphate, mM- MgSO_4 , 0.03M-substrate and mitochondria in a total volume of 3 ml. HCN, when present, was mM. Temperature, 20°.

Substrate	Young mitochondria		Mature mitochondria	
	Control	Inhibition (%)	Control	Inhibition (%)
Succinate	332	85	4650	27
L-Malate	77	11	2090	64
α -Oxoglutarate	97	—	1540	—
Citrate	—	—	1080	36

Oxidation of intermediates of the citric acid cycle and inhibition by cyanide

The rapid rates at which *Arum* mitochondria oxidized intermediates of the citric acid cycle are shown in Table 2, together with the effect of cyanide on some of the systems. The activities of the mature mitochondria were exceptionally high. The mean value from thirteen experiments at 20° for the oxygen uptake with succinate as substrate was 4060 $\mu\text{l. of O}_2/\text{mg. of N/hr.}$, which may be compared with 2190 $\mu\text{l. of O}_2/\text{mg. of N/hr.}$ at 37° for liver mitochondria (Hogeboom, Schneider & Palade, 1948). The activities of mitochondria from the young spadices were always lower. Comparison of the rates of respiration of succinate by mitochondria from young spadices collected at different stages in the season suggested that there is a steady increase in activity as the spadix grows and matures. Only the extreme values are given in Table 2 since Simon (1957) has already published similar results. The same conclusion is reached whether the activities are expressed in terms of mitochondrial nitrogen (as in Table 2) or of weight of fresh spadix (Simon, 1957). At the same time as the rate of respiration of succinate increased, the inhibition by mM-cyanide decreased. Thus the activity of the cyanide-stable succinate-oxidation system increased even more rapidly than the total activity. Simon (1957) made a similar observation but the change in cyanide inhibition was smaller in his experiments (from 65% with young mitochondria to 55% with mature mitochondria). In the present work the average inhibition was 30% in 10 different preparations of mature mitochondria, which falls between the value of 6% obtained by James & Elliott (1955) and 55% by Simon (1957).

When malate was the substrate the cyanide inhibition behaved in the opposite manner, increasing as the spadix matured. However, again the cyanide-stable rate of oxidation increased, although not so rapidly as when succinate was the substrate.

Fig. 2. Effect of cytochrome *c* concentration and pretreatment with water on the succinic-cytochrome *c* reductase activity of mature *Arum* mitochondria. ●, Mitochondria were suspended in 0.25M-sucrose before the experiment, and 0.20M-sucrose was present in the reaction mixture; ○, mitochondria suspended in 0.25M-sucrose were diluted ten times with water and kept for 92 min. at 0° before starting the reaction, and sucrose was absent from the reaction mixture. Other components of the reaction mixture were 0.05M-phosphate ($\text{KH}_2\text{PO}_4\text{--Na}_2\text{HPO}_4$), pH 7.1, 0.33 mM-adenosine triphosphate, mM- MgSO_4 , 0.03M-sodium succinate, mM-HCN, oxidized cytochrome *c* as indicated in the Figure and mitochondria in a total volume of 3 ml.

Effects of antimycin A

Succinic-cytochrome c reductase. The activity of this enzyme has been measured only in mitochondria from the mature spadix. The rates were

low unless the mitochondria were first treated with hypotonic solutions. When a mitochondrial suspension in 0.25M-sucrose was diluted ten times with water, and kept at 0° for half an hour, the reaction velocity was increased several-fold. Pretreatment in water for longer periods produced only small further increases. When the reaction was started by addition of 0.1 ml. of mitochondrial suspension to the Beckman cell, sigmoid progress curves were sometimes obtained. The reason for the lag phase has not been ascertained, but it is unlikely to be due to reoxidation of cytochrome *c* by cytochrome *c* oxidase because mM-cyanide was present in the reaction mixture. The rate was linear when the reaction was started by the addition of succinate to the mitochondria which had been incubated with the reaction mixture for 5 min. at room temperature. The rate was dependent on the concentration of cytochrome *c*. The effects of pretreatment with water and cytochrome *c* concentration are shown in Fig. 2. In this experiment the reaction was started by addition of mitochondria, and the linear portions of the progress curves were used. The succinic-cytochrome *c* reductase activity at infinite cytochrome *c* concentration after pretreatment with water was 637 μ l. of O₂/mg. of N/hr. The uptake of oxygen of the same mitochondrial preparation (without water pretreatment) in the presence of succinate was 3900 μ l. of O₂/mg. of N/hr. The succinic-cytochrome *c* reductase could therefore account for only 16% of the uptake of oxygen, but it is not

certain that the hypotonic treatment used completely activated the reductase system.

Antimycin A was found to cause 98% inhibition of the succinic-cytochrome *c* reductase at a concentration of approx. 0.3 μ g. of antimycin/mg. of mitochondrial nitrogen (Table 3).

Respiration of succinate and malate. The effects of various concentrations of antimycin on the oxidation of these substrates are shown in Table 4. The

Table 3. *Effect of antimycin A on succinic-cytochrome c reductase of mature mitochondria*

Each reaction cell contained 0.2M-sucrose, 0.05M-phosphate (KH₂PO₄-Na₂HPO₄), pH 7.1, 0.33 mM-adenosine triphosphate, mM-MgSO₄, 0.03M-sodium succinate, 52.1 μ M-oxidized cytochrome *c*, mM-HCN and mitochondria in a total volume of 3 ml. Antimycin A was added in the form of a solution in 96% ethanol. The final concentration of ethanol was made 0.96% (v/v) in all cells. 1 mg. of mitochondrial N corresponded to 1.49 g. of fresh spadix. The rate in the absence of succinate was 0.8% of the rate in its presence. This endogenous rate was inhibited 50% by antimycin (0.0493 and 0.493 μ g./mg. of N), and was not subtracted in calculating the percentage inhibition given below.

Concn. of antimycin A (μ g./mg. of N)	Inhibition (%)
0.0493	0
0.148	65
0.493	98
1.48	98
14.8	98

Table 4. *Effect of antimycin A and cyanide on respiration of succinate and malate*

Reaction mixtures were the same as in Table 2. Antimycin A was added in the form of a solution in 96% ethanol. The final concentration of ethanol was made 1% (v/v) in Expt. 1, and 0.77% in Expts. 2 and 3. The ethanol alone caused 20% inhibition in Expt. 1, 9% in Expt. 2 and 11% in Expt. 3. These are not included in the percentage inhibitions given below. 1 mg. of mitochondrial N corresponded to 3.21 g. of fresh spadix in Expt. 1, 1.31 g. in Expt. 2 and 1.22 g. in Expt. 3.

Expt. no.	Age of spadix	Substrate	Concn. of antimycin A (μ g./mg. of N)	HCN (mM)	Inhibition (%)
1	Young	Succinate	0.089	-	2
			0.445	-	39
			0.84	-	38
			8.9	-	47
			0	+	51
			0.89	+	56
2	Mature	Succinate	0.655	-	1
			1.31	-	7
			13.1	-	10
			218	-	40
			0	+	33
			13.1	+	40
3	Mature	L-Malate	0.122	-	1
			0.612	-	5
			6.12	-	10
			61.2	-	28
			0	+	56
			6.12	+	64

inhibitions were larger for the young mitochondria than the mature, but were always far from complete. The most significant concentration is about $0.3 \mu\text{g./mg.}$ of N, which gave maximal inhibition of the succinic-cytochrome *c* reductase. At this concentration there was no appreciable inhibition of the uptake of oxygen of the mature mitochondria with either succinate or malate as substrate.

Simon (1957) has also examined the effect of antimycin on the succinic oxidase system of mature *Arum*-spadix mitochondria. In contrast with the results described above he found that both mm-cyanide and antimycin in a concentration of about $3 \mu\text{g./g.}$ of fresh tissue gave about 50% inhibition, the antimycin inhibition being sometimes slightly higher than that of cyanide.

Spectroscopic experiments. When succinate is added to a concentrated suspension of mature *Arum*-spadix mitochondria the cytochromes become rapidly reduced and can be reoxidized by cooling to 0° and vigorously shaking with air (Bendall & Hill, 1956). At room temperature only a partial reoxidation of the *b* components can be obtained. However, when antimycin A ($1 \mu\text{g./g.}$ of spadix) was added to the suspension cytochromes *a* and *c* could be oxidized at room temperature. Most of the *b* components were also oxidized but a faint broad band remained at $560 \text{ m}\mu$ (Fig. 1*g*). On standing, the cytochromes fairly rapidly became reduced again. It is concluded from this experiment that antimycin inhibited the reduction of cytochromes *a* and *c*, as in heart-muscle preparations (Chance, 1952) and liver mitochondria (Chance & Williams, 1955), but did not prevent the oxidation of a large proportion of the *b* components, as it does in the animal systems. The reduction of cytochromes *a* and *c*, which still occurred after the addition of antimycin, was probably due to traces of endogenous substrate and occurred after all the dissolved oxygen had been used up by the active antimycin-insensitive succinate-oxidation system.

The above experiment was repeated with a mitochondrial suspension in 50% (v/v) glycerol. In the presence of succinate, antimycin and air a faint broad band was again visible at $560 \text{ m}\mu$. The suspension was then cooled in liquid nitrogen and the band was seen to split into three components of equal intensity at 563, 558 and $553 \text{ m}\mu$ (Fig. 1*h*). The suspension was then thawed and sodium dithionite added. Immediately cytochromes *a* and *c* became reduced and the *b* band intensified to its normal value.

The effect of antimycin A on the cytochrome system of the young mitochondria was qualitatively similar to its effect on the mature particles. This was also true of mitochondria from the spadix of *A. neglectum*.

When a concentrated suspension of washed

mature mitochondria was allowed to stand at room temperature without any added substrate the cytochromes became reduced within a few minutes owing to endogenous substrate. With this low concentration of substrate it was possible to obtain complete oxidation of the *b* components of cytochrome in the presence of mm-cyanide, and the *a* and *c* bands were seen alone at 603 and $551 \text{ m}\mu$. When this suspension was cooled in liquid nitrogen the only α -bands visible were those of cytochrome *a* at $600 \text{ m}\mu$, and cytochrome *c* at $549 \text{ m}\mu$, with a faint satellite band on the short-wavelength side. This indicated that it is possible to oxidize the components with bands at 563, 558 and $553 \text{ m}\mu$ in the presence of cyanide.

Since it is known that cytochrome *b₇* can be oxidized directly by oxygen or by a pathway not involving cytochrome *c* oxidase (Bendall & Hill, 1956), it is probable that the *b* component which remains oxidized in the presence of antimycin A is *b₇*. The three bands which are visible in the presence of antimycin at the temperature of liquid nitrogen cannot be definitely identified yet, and their nature will be discussed later. Since antimycin inhibits the oxidation of cytochrome *b* in animal systems (Chance, 1952; Chance & Williams, 1955), it is probable that one of the components in *Arum* which remains reduced in the presence of antimycin is cytochrome *b*. This suggested that comparison of the intensity of the band at $560 \text{ m}\mu$ in the presence of succinate, antimycin and air, with its intensity in the presence of dithionite, would give a measure of the amount of cytochrome *b₇* relative to that of the other components absorbing at $560 \text{ m}\mu$, including cytochrome *b*. The results of such an experiment with both young and mature mitochondria are shown in Table 5. The proportion of the total absorption at $560 \text{ m}\mu$ due to cytochrome *b₇* was 38% in the young mitochondria and 60% in the mature. It is noteworthy that in the same preparations the difference in the rates of respiration of succinate in the presence of antimycin was many times larger than the difference in *b₇* content.

Respiration of the whole tissue

James & Beevers (1950) found that mm-cyanide had no appreciable effect on the respiration of slices of *Arum* spadix. With the youngest spadices examined they obtained only an 11% inhibition. This result was surprising, since the rate of respiration of succinate by mitochondria from young spadices can be inhibited as much as 80% and because young growing plant tissues are usually more sensitive to cyanide than old tissues. It was considered necessary to check their result because their method of determining the effect of cyanide was not entirely satisfactory. To avoid the

Table 5. Concentrations of cytochrome b_7 and total b components in young and mature mitochondria

Concentration of cytochrome b_7 was obtained by subtracting the intensity of the band at $560\text{ m}\mu$ in the presence of excess of succinate, antimycin and air, from its intensity in the presence of $\text{Na}_2\text{S}_2\text{O}_4$. The antimycin concentration was $2.2\text{ }\mu\text{g./mg. of N}$ for the young mitochondria, and $3.9\text{ }\mu\text{g./mg. of N}$ for the mature mitochondria. The conditions for the measurement of rates of uptake of oxygen were as in Table 2.

Age of spadix	Concn. of cytochrome b_7 ($\mu\text{moles of haem/g. of N}$)	Total concn. of b components ($\mu\text{moles of haem/g. of N}$)	Rate of oxidation of succinate ($\mu\text{l. of O}_2\text{/mg. of N/hr.}$)
Young	0.93	2.45	280
Mature	1.53	2.55	3470

Fig. 3. Effect of mm-HCN on the respiration of two young spadices of *Arum maculatum*. Readings were taken every 5 min. Temperature, 20° .

difficulty of controlling the cyanide concentration in the manometer vessels while simultaneously absorbing the respiratory carbon dioxide, they soaked the slices in a cyanide solution for 2 hr. and then transferred them to manometer vessels containing potassium hydroxide in the centre well and no cyanide. The validity of this method depends on a slow rate of diffusion of hydrogen cyanide out of the slices and into the alkali in the centre well of the manometer flask. The rate of diffusion was not determined, but the respiration of a yeast suspension treated in the same manner was still completely inhibited 1 hr. after it was put in the manometer flasks. The experiment was therefore repeated with mm-cyanide in the main compartment of the manometer vessel and with a calcium cyanide-calcium hydroxide mixture (Robbie, 1948) in the centre well to absorb carbon dioxide and control the cyanide concentration. The cyanide stimulated the respiration by about 50%.

A similar result was obtained with the whole sterile appendages of young spadices, each appendage being placed in the centre well of a manometer flask. In this case the respiration of a spadix in the absence of cyanide was measured first, and then the spadix was transferred to a second manometer flask containing 0.1 ml. of mm-hydrogen cyanide in the centre well and 3 ml. of the appropriate calcium cyanide-calcium hydroxide mixture in the main compartment. The rate of oxygen uptake im-

mediately increased to more than double the original value and continued steadily for 40–60 min. (Fig. 3). The spadix was then returned to the manometer vessel containing no cyanide but 20% potassium hydroxide in the main compartment. The rate of respiration slowly decreased but did not reach the original value during the course of the experiment. The Q_{O_2} ($\mu\text{l. of O}_2\text{/mg. dry wt./hr.}$) in the absence of cyanide was 2.1 at 20° .

The effect of cyanide on the mature spadix has not been redetermined.

DISCUSSION

Cytochrome components

No figures for cytochrome concentrations in mitochondria from sources other than *Arum* are apparently available in the literature, except for the relative concentrations given by Chance & Williams (1955). In the materials examined by these workers the cytochrome b concentration has always been found to be rather less than that of c . It may be calculated from the data given by Slater (1949) that a Keilin & Hartree (1947) heart-muscle preparation contains one to two times more b cytochrome, and approximately ten times more cytochrome c , than mature *Arum* mitochondria. On the other hand, the activity of the succinic oxidase system of *Arum* mitochondria is about four times greater than that of the heart-muscle preparation.

Arum mitochondria have been shown to contain cytochromes a , a_3 , b_7 and c . In addition, a triple-banded spectrum, which probably cannot be ascribed to any of the above components, is visible in the presence of succinate, antimycin A and air. Of these three bands, the one at $553\text{ m}\mu$ may be due to cytochrome c_1 . The properties of cytochrome c_1 in heart-muscle preparations have been fully described and discussed by Keilin & Hartree (1955). It has been shown to be widespread in the animal kingdom and also occurs in yeast, but has not yet been described in higher plants apart from some indirect evidence obtained by Martin & Morton (1957). The evidence which suggests that the band

observed in *Arum* mitochondria at -196° at $553\text{ m}\mu$ is due to cytochrome c_1 is as follows:

(i) The position is close to that described by Keilin & Hartree (1955), who give $553\text{--}554\text{ m}\mu$ at room temperature and $551\text{--}552\text{ m}\mu$ at liquid-air temperature. The difference may not be serious since it could be due to error in the estimation of the position. Thus the band appeared at $553\text{ m}\mu$ in a heart-muscle preparation at -196° according to my estimation. It is also possible that small differences in technique, particularly in the nature of the suspending medium which was used, could cause variations in the band displacement at liquid-air temperature.

(ii) The *Arum* component was found to be thermolabile, as is heart-muscle c_1 .

If cytochrome c_1 plays an essential role in the electron-transport system of mitochondria then it may be expected to occur in plants, since the fundamental similarity in function between animal and plant mitochondria has been established.

The evidence against identification of this component as cytochrome c_1 is that it is apparently auto-oxidizable (i.e. its oxidation can be observed in the presence of cyanide) and its reduction is not inhibited by antimycin. The former observation could be explained by supposing that cytochrome c_1 can be oxidized through cytochrome b_7 or some other cyanide-insensitive oxidase as well as through cytochrome c oxidase. The latter objection is more serious since it suggests a fundamental difference in the electron-transport system in the neighbourhood of the antimycin-inhibited factor between *Arum*-mitochondria and heart-muscle preparations. A definite conclusion cannot be reached until antimycin inhibition is better understood.

It is likely that one of the remaining two bands observed at -196° in the presence of antimycin is due to ordinary cytochrome b , because in animal systems the oxidation of cytochrome b is inhibited by antimycin (Chance, 1952; Chance & Williams, 1955). The third component remains unidentified. Since it has not been observed in animals it is possible that it does not play an essential part in the electron-transport system of the mitochondria, but is rather a haemochromogen precursor or degradation product of the cytochromes which for some reason tends to accumulate more in plants than in animals.

The differences in the cytochrome components of mitochondria from the young and mature spadices lie in the relative concentrations of b components. Whereas the mature mitochondria contain a relatively large amount of cytochrome b_7 , the spectrum of the young mitochondria more closely resembles those of yeast and other plant mitochondria. The succinate-oxidation system insensitive to cyanide and antimycin increases more

than ten times as the spadix matures, whereas the b_7 content only doubles. The significance of these differences is obscure because cytochrome b_7 is only one component in an electron-transport chain, and the activities of the other components have not been determined.

Respiratory chain in Arum mitochondria

Arum mitochondria possess the normal system for electron and hydrogen transport from substrates to oxygen via cytochromes b , c , a and a_3 and possibly c_1 . In addition they possess at least one active alternative pathway which is not appreciably affected by antimycin, and at most only partially inhibited by mm-cyanide.

The succinic-cytochrome c reductase of *Arum* mitochondria was found to be almost completely inhibited by antimycin, and Simon (1957) has shown that mm-cyanide gives essentially complete inhibition of *Arum* cytochrome c oxidase. The uptake of oxygen by mature mitochondria with succinate as substrate was not significantly inhibited by a concentration of antimycin which completely inhibited the succinic-cytochrome c reductase. It is therefore probable that the oxidation system insensitive to cyanide and antimycin is capable of mediating almost all the uptake of oxygen for the oxidation of succinate to fumarate with mature mitochondria. Cyanide inhibited the respiration of succinate to a significantly greater extent than did the critical concentration of antimycin A. It is likely that the explanation of this lies partly in that a proportion of the observed uptake of oxygen in the presence of succinate is due to oxidation of malate formed from the succinate, and that oxidation of malate is inhibited to a large extent by cyanide.

The oxidation of a variety of substrates by intact liver mitochondria is completely inhibited by antimycin (Potter & Reif, 1952; Copenhaver & Lardy, 1952). This may be taken to mean that the cytochrome c -cytochrome oxidase pathway is completely inhibited; if this is also true of *Arum* mitochondria, the lack of inhibition of the respiration of malate by antimycin with mature mitochondria indicates that an alternative oxidation pathway plays a significant role in oxidation of malate. However, this is uncertain because of the large difference between the effects of cyanide and antimycin on oxidation of malate.

The larger effects of cyanide and antimycin on oxidation of succinate by young mitochondria indicate that as the spadix grows the alternative oxidase system increases relative to cytochrome c oxidase. This is supported by the demonstration by Simon (1957) that the cytochrome c oxidase activity decreases relative to the rate of respiration of succinate during this time.

Hackett (1957) has recently shown the presence of a pathway insensitive to cyanide and antimycin in mitochondria from the flowers of the skunk cabbage (*Symplocarpus foetidus*). Whether this system is significant in mitochondria from tissues other than aroid spadices is not yet known.

Function of the mitochondria in the respiration of the tissue

It is possible to estimate the importance of the mitochondria in the respiration of the whole tissue by a comparison of the rate of respiration of succinate by the mitochondria with the uptake of oxygen of the spadix. Even though no attempt at a quantitative recovery of mitochondria was made, the rate of oxidation of succinate by the mature mitochondria corresponded to a Q_{O_2} of 17 at 20°. According to James & Beevers (1950) the Q_{O_2} of the spadix may reach a value of 30 at 30°. It seems likely therefore that the mitochondria *in vivo* could account for all the rapid uptake of oxygen of the tissue. Furthermore, it was found that after centrifuging a concentrated homogenate of the mature spadix for 1 hr. at 100 000 g, only 1–2 % of the uptake of oxygen of the homogenate remained in the supernatant.

The Q_{O_2} of young mitochondria with succinate as substrate was variable, but was of the same order as the Q_{O_2} of the whole tissue. It is therefore surprising that whereas oxidation of succinate might be inhibited 80 % by cyanide, the respiration of the whole tissue was not inhibited at all, but stimulated. This apparent contradiction is unexplained.

SUMMARY

1. The cytochromes of mitochondria from young and mature spadices of *Arum maculatum* were compared. It was found that cytochrome *b*₇ occurs in young mitochondria, but in a smaller concentration relative to that of cytochrome *c* than in the mature.

2. A method is described for measuring the concentration of cytochromes *a* + *a*₃ in tissue preparations, by comparison with a chlorinated resorufin derivative. The concentrations of *a*, *b* and *c* cytochromes in *Arum* mitochondria were estimated.

3. The uptake of oxygen by *Arum*-spadix mitochondria in the presence of succinate or malate increased as the spadix matured.

4. Cyanide and antimycin A only partially inhibited the respiration of succinate and malate. Antimycin A gave smaller inhibitions than cyanide. It is suggested that there is an increase in the importance of the pathway insensitive to cyanide

and antimycin relative to the cytochrome *c* oxidase pathway as the spadix matures.

5. The succinic-cytochrome *c* reductase of mature mitochondria was completely inhibited by antimycin A.

6. Antimycin A inhibited the oxidation of cytochromes *b* and two components with bands at 553 and 563 mμ. The oxidation of cytochrome *b*₇ was not inhibited.

7. The respiration of the whole young spadix was stimulated by mM-cyanide, even though the oxidation of succinate by the mitochondria could be inhibited as much as 80 %.

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