CYTOCHROME COMPONENTS IN THE SPADIX OF ARUM MACULATUM

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(With 1 figure in the text)

Introduction

The discovery of James and Beevers (1950) that the rapid oxidation processes characteristic of spadix tissue were not significantly influenced by cyanide suggested that a cytochrome system was not involved. The subsequent work of Hackett and Simon (1954) showed that part of the respiratory activity could be recovered from a particulate fraction obtained from the tissue homogenate; also they found that the particles were able to oxidize succinate as well as other carboxylic acids. Recently James and Elliott (1955) have obtained further evidence leading to the conclusion that the particles from the spadix resemble the mitochondria obtained from many plant and animal tissues in their ability to oxidize components of the tricarboxylic acid cycle but differ in respect to the lack of inhibition shown by the *Arum* particles in presence of cyanide. Since the succinoxidase system in the particulate fraction from many types of cell has so far always been found to be associated with the presence of cytochrome components it was important to see whether the spadix tissue was exceptional in this respect.

Methods and Materials

Inflorescences of *Arum maculatum*, at a stage just prior to the opening of the spathe, were collected from wild colonies near Cambridge during April, May and June 1954 and 1955. The experimental material was the sterile head of the spadix, after removal of the papillae and coloured cell contents of the epidermis.

Spectroscopic observations were made with a Zeiss microspectroscope, both on longitudinal sections of suitable thickness, and on particulate fractions. The relative intensities of the absorption bands were estimated by comparison with the α-bands of yeast cytochrome using the spectrocolorimeter of Hill (1936). A 12 per cent suspension of bakers' yeast in concentrated sucrose was used in the colorimeter cup.

Particulate fractions were obtained by grinding up to about 40 gm. of tissue in a chilled mortar with 45 ml. of ice-cold 0.25 M sucrose, straining the brei through muslin, and differentially centrifuging the resulting homogenate in an International refrigerated centrifuge model PR-1 and a Spinco preparative ultracentrifuge (used only for the final centrifuging at 100,000 *g*). The homogenate was first centrifuged for 5 min. at 1500 *g*, which removed principally starch. The mitochondria were then precipitated by centrifuging the supernatant for 15 min. at 6500 *g* and were washed by suspension in 0.25 M sucrose and recentrifugation. The mitochondrial pellet was pale brown. The yellow supernatant was further centrifuged for 30 min. at 21,000 *g* giving a yellow pellet containing a mixture of mitochondria and microsomes, which was discarded. The

microsomes were finally precipitated by centrifuging the supernatant for 1 hour at 100,000 *g*. The gelatinous precipitate was orange and transparent. It was washed once by suspension in 0.25 M sucrose and recentrifuging. For spectroscopic observations the mitochondrial and microsomal pellets were suspended in 0.2 M sucrose containing 0.05 M phosphate pH 7.1. It was found convenient to prepare the mitochondria from 10 gm. of tissue for observation of the cytochrome spectrum. If the mitochondria were suspended in a total volume of 2 ml. and examined in a 1 cm. cell, a strong spectrum could be observed. For a preparation of microsomes a convenient amount of tissue was found to be 30-40 gm., when the preparation was examined in the same volume and thickness of suspension.

The terms mitochondria and microsomes are used for convenience only, and are not intended to imply definitions of the nature of the particulate fractions.

Results and Conclusions

Living Tissue

The fresh spadix tissue shows in a slice 1 mm. thick, when air is excluded, a spectrum similar to that seen in many chlorophyll-free plant tissues (Hill and Scarisbrick, 1951, fig. 1). For example the intensity of the absorption spectrum is similar to that of a considerably thicker (7 mm.) slice of the phloem storage tissue of a parsnip root. In the spadix the band of cytochrome *a* is visible while components *b* and *c* are represented by a single fused band. However, this band is markedly asymmetric, being denser on the long wavelength side, and was seen to be nearly of the same intensity as that of the α-band of cytochrome *b* observed in the same thickness of a sample of pressed bakers' yeast. In the yeast the band of component *a* then appeared to be about three times as strong as in the spadix tissue. When examined in air the slice of spadix showed a little of the band *a* and the second band was partly reduced in intensity and appeared to be at about 560 mμ. It is concluded that the sterile tissue of the spadix has a relatively high concentration of cytochrome components of the same order as that in the bakers' yeast.

Mitochondria

The unwashed mitochondria showed in the absence of air a spectrum similar to that of the intact spadix tissue — an *a*-band at 605 mμ, a strong *b*-band at 560 mμ, and in addition a faint *c*-band at 552 mμ. Even when *b* was oxidized *c* was at a slightly longer wave length than purified heart muscle *c*. When a strong suspension was shaken with air in the cold the absorption bands temporarily disappeared but soon reappeared on standing. If cyanide was added in a concentration of approximately 10^{-3} M and the suspension shaken with air the band at 560 mμ became faint and indistinct while the bands of *a* and *c* remained unaltered. On standing, the strong band at 560 mμ reappeared.

In the washed mitochondrial preparations the cytochrome components were immediately reduced in the presence of succinate. However, even when the reduction was carried out *in vacuo* the intensity of the *b*-band was only about 60 per cent of that visible on reduction by $Na_2S_2O_4$. It was not possible to observe the oxidation of the cytochrome components by shaking the strong suspension with air in the presence of succinate at room temperature. The oxidation could be observed if the reduction rate was diminished by cooling the suspension to 0°. When cyanide was added to the suspension in the presence of succinate at the lower temperature the bands *c* and *a* remained on shaking in air while the band at 560 mμ was greatly reduced in intensity.

The addition of excess ethyl urethane to the suspension of mitochondria in the presence of succinate delayed the reduction of the cytochrome components after shaking in air. On standing, the first bands to reappear were *a* and *c* followed by the reappearance of the band at 560 mμ. Thus the spadix mitochondria showed a marked difference in behaviour from that shown by living yeast or by muscle preparations, where component *b* remains in the reduced state in the presence of urethane and succinate even when *a* and *c* are completely oxidized (Keilin, 1925; Keilin and Hartree, 1939). In the presence of urethane and cyanide together there was no disappearance of bands *a* and *c* but the band at 560 mμ completely disappeared when the suspension was shaken in air.

When L-malate was added to a washed mitochondrial suspension *in vacuo*, the *a*-, *b*-, and *c*-bands were immediately reduced. However the *b*-band at 560 mμ was less dense than when succinate was used as the reducing agent. On shaking with air at 0° the bands disappeared, but in the presence of approximately 10^{-3} M cyanide *a* and *c* remained reduced while *b* was to a large extent oxidized, just as in the presence of succinate.

It is concluded from these observations that the mitochondria obtained from the sterile head of the *Arum* spadix contain a relatively high proportion of a cytochrome *b* component which has properties different from those of the normal component *b* of cytochrome. This new *b* component, α-band at 560 mμ, will be referred to in what follows as b_7. When a strong suspension of the mitochondria was examined in the presence of $Na_2S_2O_4$ so that the α-band of b_7 was very intense an absorption band was observed at 529 mμ. This was taken to be mainly due to the β-band of b_7.

It may also be concluded that the particles also contain a normal cytochrome system, at least in so far as the presence and behaviour of components *c* and *a* are concerned. There is, however, a relatively small amount of component *c* as compared with the component *a*. The component b_7 is rapidly oxidized in the presence of air and the oxidation is unaffected by cyanide. In an atmosphere of carbon monoxide the absorption band of the reduced b_7 shows no change. While in the unwashed mitochondrial preparations reduction of cytochrome b_7 is due to the presence of natural substrates, in washed preparations, where reduction of b_7 is slow, immediate reduction occurs when succinate and L-malate, which are known to be oxidized by the preparation, are supplied.

It was found that the cytochrome components in the spadix mitochondria would react reversibly with the ferri-ferro oxalate system (E'_0 = zero pH 6-7, Michaelis and Friedheim, 1931). A suspension of the mitochondria was placed in a vacuum tube in the presence of 10^{-2} M ferric oxalate with excess of potassium oxalate (10^{-1} M). The suspension was then titrated in the absence of air with small successive additions of a 0.1 M ferrous sulphate solution added through the side-tube as described by Hill (1936) (cf. Hill, 1954). As soon as a trace of ferrous salt was added the components *a* and *c* became reduced, in accordance with the values of the oxidation-reduction potentials determined for these components from animal tissues. When further small additions were made a shading appeared to the longer wavelength side of the cytochrome *c* α-band, followed by a distinct though faint band at 563 mμ in the position of component *b* of cytochrome. When Fe^{2+} was equal to Fe^{3+}, (E_h = zero), the *b*-band was slightly stronger and appeared between 563 and 560 mμ. This was taken to represent a mixture of *b* and b_7 in equal amounts. The half reduction of b_7 was estimated to correspond to a ratio of Fe^{3+}/Fe^{2+} corresponding to a potential of −0.03 volt. Thus the E'_0 of b_7 at pH 7 is more negative than the E'_0 of cytochrome *b*, which is zero at pH 7 when measured with the iron oxalate system (Hill, 1954). It may be concluded from this experiment that

the spadix mitochondrial preparation contains a normal cytochrome system (in addition to cytochrome b_7) and that this system is markedly deficient in components *b* and *c* as compared with component *a*, in terms of the relative intensities of the absorption bands.

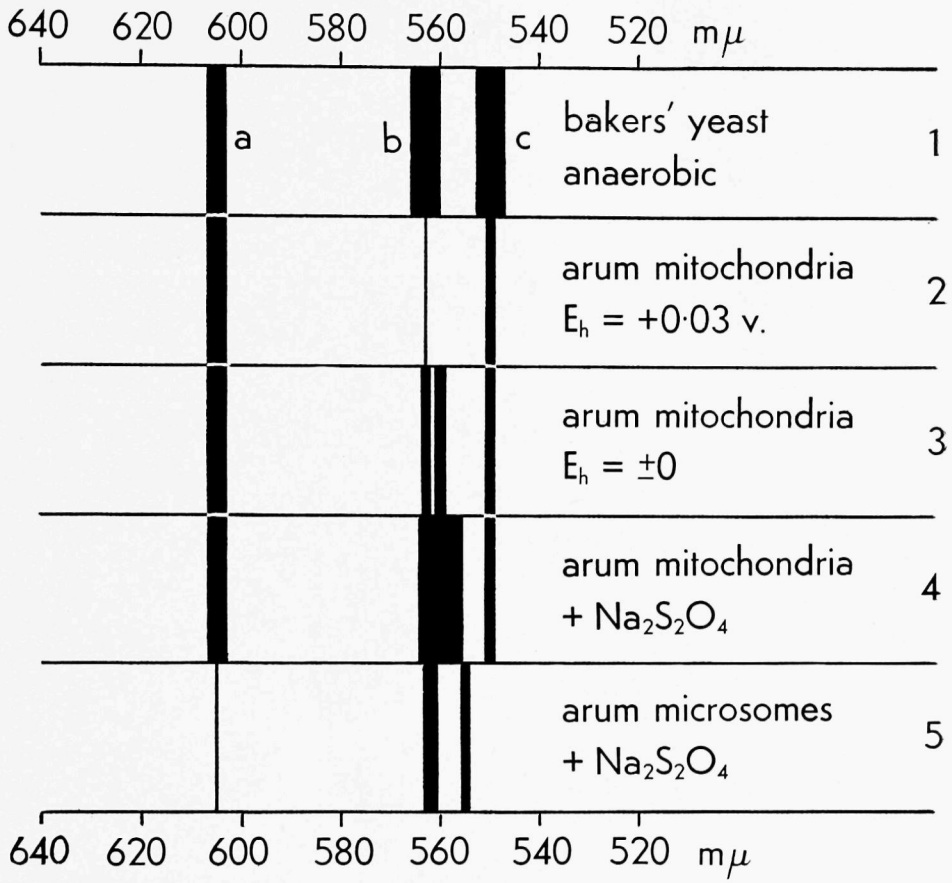

Fig. 1. Diagram showing certain cytochrome components, α-bands only, visible in the particulate fractions from the sterile head of the *Arum* spadix, compared with living yeast. For explanation see text. (Note: In 3 the band *b* is shown double to represent a mixture of two components, *b* and b_7; actually a diffuse band is observed with the centre in the position of the narrow white line. In 2 the band of *b* at 563 mμ alone could be observed and absorption due to b_7 is not represented on the diagram.)

In the figure 2, 3, 4 show in a diagrammatic form three of the stages in reduction of the cytochrome components together with a similar diagram 1, of the α-bands of reduced cytochromes *a*, *b*, and *c* in bakers' yeast. The widths of the absorption bands have been made approximately proportional to their relative intensities. The diagram thus represents an interpretation of the absorption spectra, and is not intended to be a pictorial representation of them. The band *a* in the spectrum of the yeast has been made equal to that in the spadix mitochondria to aid comparison with the other components.

The position of the α-band of b_7, about 560 mμ, is very near to that of cytochrome b_3, α-band 559.7 mμ, described by Hill and Scarisbrick (1951). Cytochrome b_3, however, was found by them to be readily reduced both by ascorbic acid and by leuco-methylene blue; it was also reduced by illuminated chloroplast preparations in the presence of air. This would indicate that cytochrome b_3 has a more positive potential than b_7, ($E'_o = -0.03$

v. pH 7). An experiment with a preparation of cytochrome b_3 from pea leaves led to the conclusion that the characteristic potential at pH 7 was approximately +0.04 v. when the extent of reduction was measured in relation to the iron oxalate system. Cytochrome b_7 seems also to be distinguished by being firmly attached to the insoluble fraction, while b_3 can readily be separated in a soluble form. It is thus concluded that while b_3 and b_7 can with difficulty be distinguished by the position of the α-bands, the oxidation-reduction potential and the difference in solubility readily serve to distinguish them.

The presence of cytochrome b_7, in easily visible quantity, seems to be confined to the sterile tissue of the spadix. A preparation of mitochondria from the female flowers of *A. maculatum* showed a normal cytochrome spectrum like that of bakers' yeast.

Microsomes

The absorption spectrum of the reduced microsomes may be clearly seen by examining the unwashed orange pellet with a Zeiss microspectroscope. The spectrum seen was similar to that described by Davenport (1952) in the chloroplast fraction from etiolated barley leaves examined in the presence of $Na_2S_2O_4$. In a strong suspension of the *Arum* microsomes the haemochromogen α-band was also double showing components at 562 and 555 mμ, the band at 562 mμ being slightly denser than the other (Figure, see 5). The band at 562 mμ approximately corresponded to the position of cytochrome b_6 characteristic of chloroplasts (Hill, 1954) and the band at 555 mμ, while corresponding in position with cytochrome *f*, was shown to belong to a different component as will be explained later. A trace of the α-band of cytochrome *a* was also visible. When the washed microsomal pellet was resuspended in sucrose-phosphate the bands described above disappeared, but a faint diffuse band appeared at 570 mμ. The reduced spectrum reappeared when the suspension was evacuated in a Thunberg tube, or on addition of $Na_2S_2O_4$. It was found, however, that the component at 555 mμ could not be reduced by addition of excess of ferrocyanide; thus the band at 555 mμ seems to correspond to a haem compound of a more negative potential than either cytochrome *f* or cytochrome *c*. It was estimated that the half-reduction of both components occurred when the ratio of ferri/ferro of the oxalate system corresponded to a potential of −0.06 v. This corresponds to the potential of cytochrome b_6 as measured by Hill (1954). From this it is concluded that the microsomes contain a component similar to cytochrome b_6 but that the component at 555 mμ is different from cytochrome *f*. It is not possible to say at present whether the microsomes themselves contain cytochrome *a*, or whether the faint *a*-band is due to the presence of small amounts of mitochondria or their fragments. In addition it is concluded that in the supernatant fluid natural substrates are present which are capable of reducing the microsomal cytochromes, and that reoxidation of these cytochromes can occur in the presence of air.

The yellow pigment of the microsomes showed the properties characteristic of a mixture of carotenoids. A significant proportion of this reacted with concentrated HCl to give a blue solution characteristic of the epi-oxide type represented by the pigment violaxanthin.

DISCUSSION

It seems justified to assume that cytochrome b_7 can provide a path of oxidation alternative to the cytochrome *c*-cytochrome oxidase system. This would imply that the new *b* component is directly concerned in the cyanide stable respiration of the head of the spadix. There is no *direct* evidence so far that cytochrome b_7 is itself to be regarded as the

terminal oxidase of the cyanide stable respiration, although there is also no evidence that would negative this view. The quantity of b_7 present, the rate of its reduction in the tissue homogenate and the ready oxidation even in the presence of cyanide, which can easily be observed when the reduction rate is diminished, make it necessary to assume that a significant part of the respiration involves the oxidation and reduction of b_7. How significant the contribution is can only be measured by kinetic studies which so far have not been carried out.

Outstanding questions in the interpretation of the behaviour of cytochrome b_7 in relation to the respiration are whether it may actually replace cytochrome *b* or whether it acts by catalysing the oxidation of cytochrome *b*. The experiments with excess of urethane in the presence of succinate indicate that cytochrome *b* in the particles is rapidly oxidized under these conditions. This is in contrast to the behaviour of component *b* in yeast or in red muscle. However, the specific effect of urethane on component *b* has so far been little studied in higher plants, so that the ready oxidation of *b* in the *Arum* preparations in presence of urethane is not necessarily an indication that b_7 is oxidizing component *b*. There appears to be relatively little cytochrome *b* as compared with *a*, which might suggest that cytochrome b_7 replaces cytochrome *b*. But there is no indication that the oxidation of b_7 can occur through the cytochrome *a*-cytochrome oxidase system.

The possibility exists that cytochrome b_7 and the normal cytochrome system occur on distinct types of particle. This consideration is complicated by the results of experiments with malate; succinate reduces cytochrome b_7 to the extent of 60 per cent while malate reduces it even less, although with both substrates reduction occurs rapidly. It would be expected from the oxidation-reduction potentials that cytochrome b_7 would be reduced by malate to a greater extent than by succinate. This would suggest that the component b_7 is not linked with the oxidation of malate in all of the particles which contain b_7.

The presence of carotenoids in the microsomes, and of cytochrome components partially resembling those of etiolated barley chloroplasts, suggests that these are to be regarded as plastids. On the other hand the mode of preparation suggests analogy with microsomes isolated from animal tissues. It may indeed be that the *Arum* microsomes are related to both plastids and animal microsomes. Since the work reported above was completed, preparations of microsomes from pea cotyledons and from rat liver have been examined and found to show the double α-band, resembling the preparations from *Arum*. A double α-band has also been noted by Chance, Klingenberg, and Boeri (*Fed. Proc.* **15**, 231, 1956), in contrast to the single cytochrome component described by Strittmatter and Ball (1952, 1954) for rat liver microsomes.

Summary

(1) The sterile tissue of the spadix of *Arum maculatum* was found to be well supplied with cytochrome components. The cytochrome spectra of the living tissue and of particulate preparations have been described.

(2) A mitochondrial fraction was found to contain a normal cytochrome system with components *a*, *b*, and *c*, and in addition a large amount of a cytochrome with α-band at 560 mμ, which was termed b_7.

(3) Cytochrome b_7 did not react with carbon monoxide and was oxidized by air in the presence of cyanide. It is suggested that this may account for a significant part of

the cyanide stable respiration of the tissue, and also of the cyanide stable oxidation of succinate and L-malate by the mitochondria.

(4) The E'_0 of cytochrome b_7 was found to be approximately -0.03 v. at pH 7.

(5) A yellow microsome fraction was found to contain carotenoids, and two cytochrome components. One of these is similar to cytochrome b_6 of etiolated chloroplasts, and the other has an α-band at 555 mμ, but is not identical with cytochrome *f*.

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