A FLAVOPROTEIN FROM *ARUM* SPADIX

By W. O. JAMES and DAPHNE C. ELLIOTT

Department of Botany, Oxford

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(With 2 figures in the text)

Summary

A flavoprotein has been extracted from the mitochondria of *Arum* spadix. The prosthetic group is flavine-adenine-dinucleotide and the enzyme behaves as a DPN-specific cytochrome *c* reductase. It is also autoxidizable. Its respiratory function is discussed.

The existence of an autoxidizable flavoprotein in extracts from *Arum* spadices was observed by James and Beevers (1950) and its possible respiratory significance recognized. Extraction from the tissue in a form suitable for further investigation was not found possible; but its extraction and partial purification from separated mitochondria has now been achieved. In the following paper the method of extraction and the examination of the product are described. The enzyme proves to be a fairly stable flavoprotein with a flavine-adenine-dinucleotide (FAD) prosthetic group, a high degree of specificity for DPN (coenzyme I) and the ability to reduce cytochrome *c* rapidly. It is also autoxidizable and the reductase and terminal oxidase functions have not yet been separated.

Material and methods

Arum spadices were collected in the Oxford neighbourhood and brought immediately to the laboratory and cooled to 2° C. Spadices from unopened inflorescences and with minimal amounts of anthocyanin in the surface cells were selected.

Mitochondria were extracted from spadix samples by the method of Hackett and Simon (1954) and were washed once with 40 ml. medium.

Extraction of the flavoprotein

The mitochondrial pellet from 36 gm. spadix tissue was suspendec
extraction medium. This was made up to 15 ml. with distilled water, a
alcohol, previously cooled to −10° C., was added very slowly with c
give a final concentration of 70 per cent alcohol. The vessel was surrounded by a freezing mixture maintaining a temperature of −5° C. throughout this stage.

The alcohol-treated mitochondria were then centrifuged down at −5° C., and the precipitate taken up in 12 ml. ice-cold 0.5 M phosphate buffer, pH 7.0. Denatured protein was centrifuged off and 13 ml. of supernatant 'flavoprotein solution' was obtained.

Isolation of the prosthetic group

The mitochondrial fraction not precipitated by cold alcohol was bright yellow but did not yield any flavine on concentration. The insoluble denatured residue remaining after extraction with phosphate buffer was brought to the boil with 3 ml. water but no flavins were extracted. It therefore appeared that the flavin prosthetic group was efficiently extracted in the 'flavoprotein solution' obtained as above.

The flavoprotein was split by the following method. Ammonium sulphate was added to 10 ml. 'flavoprotein solution' until it was 0.7 saturated and the solution held in a boiling water bath for 10 minutes. After cooling it was centrifuged to clear and the supernatant (8.0 ml.) was extracted three times, with 8 ml. *p*-cresol. The combined cresol extracts amounting to 20 ml. were mixed with 40 ml. ether and then extracted with three successive 1 ml. aliquots of distilled water to give an aqueous solution of the free flavin.

Results

Identification of the prosthetic group

Chromatography

Aliquots of the flavin solution prepared as above were run in two separate types of one-way chromatogram on paper. The first was a descending chromatogram using *n*-butanol/glacial acetic acid/water in the proportions 4/1/5 by volume. The second was an ascending chromatogram in 5 per cent aqueous $Na_2H\ PO_4\ .\ 12\ H_2O$. Of a number of solvents tried these two were found to be the most useful.

Table 1. *RF values observed in* 1*-way chromatograms*

Flavin	Solvent	
	Butanol/ Acetic/Water	Aq. Sodium phosphate
FAD	0.04	0.31
Arum flavin	0.04	0.31
FMN	0.09	0.48
Riboflavin	0.37	0.24

All the spots were yellow and were strongly fluorescent in ultraviolet light. Two small spots, also fluorescent, were observed in the butanol-acetic chromatogram, at RF 0.12 and 0.17 but were not identified.

The remainder of the flavin solution was run as a band on a butanol-acetic chromatogram. The principal band was eluted and used for further tests.

An aliquot was mixed with an authentic preparation of flavine-adenine-dinucleotide and run in a second butanol-acetic chromatogram. There was no separation and the chromatogram showed a single spot only. With the aqueous sodium phosphate a spot of the preparation run beside a spot of the known FAD had an identical RF.

D-amino acid oxidase activity

D-amino acid oxidase was prepared from fresh sheep's kidneys by the method of Negelein and Brömel (1939) and the flavin removed by treatment with saturated ammonium sulphate (Davison, 1951). The protein was resuspended in pyrophosphate buffer, pH 8.3. Catalase was prepared by the method of Bonnichsen (1947).

Preparations were set up in Warburg manometer flasks containing 0.3 ml. D-amino acid oxidase suspension, 0.1 ml. catalase, 0.3 M ethanol, 0.3 ml. 0.4 M alanine (in the side

arm), 1.0 ml. pyrophosphate buffer pH 8.3 with 0.2 ml. 10 per cent KOH (in the centre well) and water to give 3.3 ml. in all. The concentration of the *Arum* flavin solution was adjusted to give an optical density equal to that of the known FAD. Flasks were then set up in which 0.3 ml. of FAD or *Arum* flavin solution replaced 0.3 ml. water. The control flasks showed little uptake of oxygen. Flasks in which the enzyme was reconstituted by addition of FAD showed an oxygen uptake about nine times faster and those with *Arum* flavin one faster still.

Table 2. *D-amino acid oxidase activity*

	μl O_2 consumed in 60 minutes. Contents of flasks as given in the text.
Control	15
With flavine-adenine-dinucleotide	140
With *Arum* flavin	708

The clear flavoprotein solution was pale yellow and had a strong pale green fluorescence in U.V. light. Both colour and fluorescence were quenched by a small addition of dithionite and both returned after a short period of shaking by hand in the presence of air. The enzyme was, therefore, autoxidizable. It was precipitated by a small addition of phosphotungstate or alcohol.

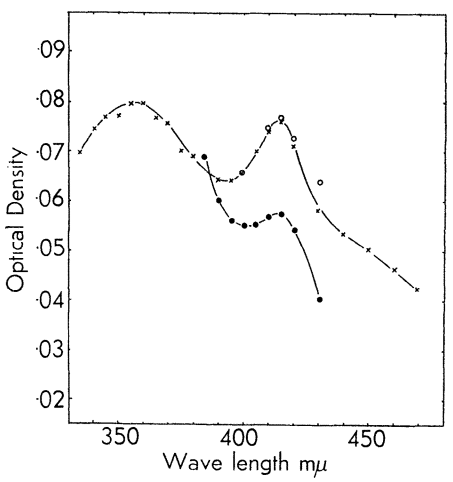


FIG. 1

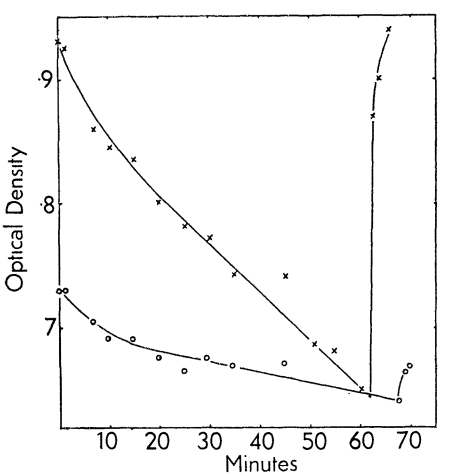


FIG. 2

Fig. 1. Absorption spectrum of *Arum* flavoprotein in the region λ = 330 to 470 m μ. There is also a much more intense peak of absorption at 260 mμ which does not change on reduction. x, initial solution; ● after reduction with dithionite; o after shaking the reduced solution in air.

Fig. 2. Optical density at λ = 340 mμ. x with DPN; o with TPN. After 62 minutes alcohol, alcohol dehydrogenase and semicarbazide were added to the DPN cuvette. After 68 minutes isocitrate+isocitric dehydrogenase was added to the TPN cuvette. The optical densities are corrected for the small dilutions due to addition of reagents.

Spectroscopic examination

The absorption spectrum of a sample of the flavoprotein solution was examined in a Hilger spectrophotometer. The spectrum observed is recorded in Fig. 1. Two peaks of absorption occurred at λ = 357 and 415 mμ. Absorption at wave lengths above 390 mμ was sharply reduced by a small addition of dithionite and was restored on shaking in air.

The position of the peak at 415 mμ differs from that of FAD which lies at 450 mμ. A sample of the prosthetic group isolated from the *Arum* flavoprotein showed a peak at 450 mμ agreeing with the free flavin. The maximum difference between the oxidized and reduced spectra of the flavoprotein lies to the right of the 415 mμ peak. In all these respects the enzyme corresponds very closely with the aldehyde oxidase of liver (Mahler, 1956; Gordon, Green and Subrahmanyan, 1939).

Reaction with coenzymes

DPN (coenzyme 1) was reduced enzymatically with alcohol and crystallized alcohol dehydrogenase; TPN (coenzyme 2) with an isocitric system. Flavoprotein was extracted from 12 gm. spadix in the usual way and taken up in 2 ml. phosphate buffer at pH 7. The oxidation of the coenzyme was followed by recording the absorption at $\lambda = 340$ mμ in a Unicam spectrophotometer with a light path of 1.0 cm. The cuvettes contained 0.5 ml. flavoprotein solution+coenzyme in phosphate buffer, pH 7, to 3.0 ml. The results are recorded in Fig. 2. Oxidation of DPN was rapid; but oxidation of TPN was only slight. After about 60 minutes 0.2 ml. ethanol, a little crystalline alcohol dehydrogenase and a little semicarbazide were added to the cuvette containing DPN. Re-reduction of the coenzyme was immediate and complete. On adding isocitric acid and isocitric dehydrogenase to the TPN cell a small increase of absorption was recorded. The flavoprotein has therefore a marked specificity towards DPN. DPN reduced with dithionite by the method of Umbreit *et al.* (1949) was also readily oxidized.

Reduction of cytochrome c

The reduction of cytochrome *c* was observed in a Beck visual microspectrometer by means of the *c*α-band at $\lambda = 550$ mμ. A depth of approximately 5 mm. was used. In a system containing 2 drops ethanol+0.1 ml. crystalline alcohol dehydrogenase solution +0.1 ml. containing 0.01 DPN (Sigma)+0.1 ml. containing 0.1 mg. cytochrome *c* (Sigma) no spectral change had occurred after 30 minutes. A parallel mixture containing 0.1 ml. *Arum* flavoprotein solution in addition caused the appearance of a heavy band at 550 mμ. This was not further intensified on addition of dithionite. The cytochrome *c* was therefore fully reduced by the flavoprotein.

Cytochrome b_7

A suspension of mitochondria obtained from *Arum* spadix by the usual procedure was examined in the Beck microspectrometer and showed a faint absorption band at 605 mμ (cytochrome *a*) and a stronger band at 560 mμ (cytochrome b_7; Bendall and Hill, 1956). Cooling and shaking the suspension in air caused the disappearance of both bands which returned again slowly on standing. Addition of dithionite darkened the bands. Malate caused partial reduction as previously observed by Bendall and Hill.

An alcohol-dehydrogenase-DPN system was set up as in the previous section and an oxidized mitochondrial suspension added. The cytochrome b_7 band at 560 mμ was fully visible after 1 minute. This did not represent any acceleration of the reduction observed with the mitochondria alone. Further addition of 0.1 ml. *Arum* flavoprotein solution did not increase the rate of reduction either. On addition of a drop of cytochrome *c* solution, a strong band appeared at 550 mμ immediately, but no band at 560 mμ, i.e. the cytochrome b_7 was not reduced by the flavoprotein directly or through cytochrome *c*.

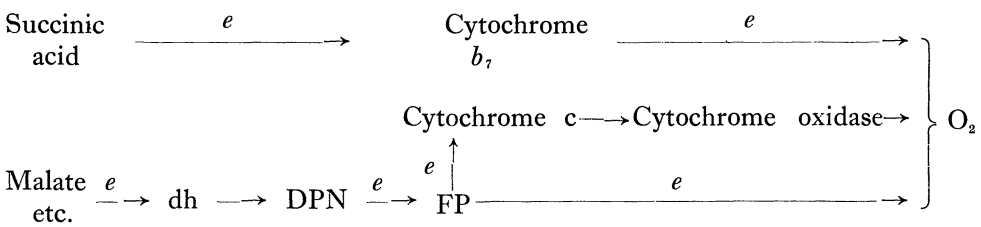
DISCUSSION

Since the rapid respiration of *Arum* spadix is not inhibited appreciably by cyanide, it was suggested by James and Beevers that an autoxidizable flavoprotein might act as terminal oxidase. The enzyme described in this paper might be capable of fulfilling the role; but we have not been able to demonstrate that its turnover is fast enough to sustain the very rapid rates involved.

In the meantime the discovery by Bendall and Hill of an autoxidizable cytochrome b_7, also unaffected by dilute cyanide, provides a second alternative. Small amounts of cytochrome *c* and cytochrome oxidase also appear to exist in the mitochondria of *Arum* spadix, and it is therefore possible that, before application of cyanide, at least part of the respiration might proceed by this route.

The flavoprotein described in the present paper is highly active in transferring electrons from DPN to cytochrome *c* and may thus be a link in the oxidation chain of malic and other acids through cytochrome *c* or, since it is autoxidizable, direct to oxygen.

At present there appear to be at least three routes by which respiratory oxidation may occur in *Arum* spadix.



dh = dehydrogenase, FP = flavoprotein.

The relative importance of the three pathways is still uncertain.

We wish to record our indebtedness and our thanks to Dr. D. D. Davies for samples of pure alcohol and isocitric cehydrogenases; and to Mr. W. F. Warren who carried out the examination of the D-amino acid oxidase activity of the flavin.

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