Rapid degradation of FAD following lysis of Neurospora crassa cells: Consequences for evaluation of flavin composition in vivo.

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Abstract
Rapid degradation of FAD following lysis of Neurospora crassa cells: Consequences for evaluation of flavin composition in vivo.
Phosphate mediated changes in fatty acid composition in *Neurospora crassa.*

From our earlier studies on the effect of inorganic phosphate on alterations in phospholipids (Nair and Chhatpar, 1983, Neurospora Neuletter 30: 11) and changes in sugar uptake in *Neurospora crassa* (Savant, Parikh and Chhatpar, 1982, Experientia 38: 310-311), we suggested that phosphate might play an important role at the membrane level with respect to uptake and permeability functions. Further, we were interested in seeing the effect of inorganic phosphate on membrane fatty acid composition since alterations in fatty acid composition have been shown to result in changes in ion permeability and enzyme activity (Davis and Silbert, 1974, Biochim. Biophys. Acta 373: 224; Dekruyff, et al., 1973, Biochim. Biophys. Acta 298: 479).

The synthetic liquid medium employed for the growth of *N. crassa* (wild type, carotenogenic) contained per litre: glucose, 50 g; trisodium citrate, 2.5 g; (NH₄)₂SO₄, 2.5 g; MgSO₄.7H₂O, 0.5 g; ZnSO₄.7H₂O, 2.5 mg; FeCl₃, 5.0 mg; CaCl₂, 10 mg; and biotin 100 pg. The pH was adjusted to 5.6. 'High phosphate' condition indicates the addition of KH₂PO₄ to the above medium. 1.0 g% whereas 'Low phosphate' condition indicates the addition of 0.01 g%KH₂PO₄ to the above medium. These phosphate conditions did not change the pH of the medium. Growth temperature was 30° C. Culture density at harvest was determined by drying mycelial mats at 50° C to constant weight: for the low phosphate culture dry mat weight was 0.24 g/50 ml flask, and for the high phosphate culture it was 0.28 g/50 ml flask.

### TABLE I

Effect of inorganic phosphate on percentage fatty acid composition in *Neurospora crassa*

| Type of fatty acid | High phosphate (KH₂PO₄:1g%) | Low phosphate (KH₂PO₄:0.01g%) |
|-------------------|-------------------------------|-----------------------------|
| C₁₄               | 18.87                         | 64.16                       |
| C₁₆               | 16.02                         | 9.17                        |
| C₁₆:1             | 6.66                          | ND                          |
| C₁₈               | 6.27                          | ND                          |
| C₁₈:1             | 6.27                          | 5.24                        |
| C₁₈:2             | 27.34                         | 17.07                       |
| C₁₈:3             | 18.58                         | 4.36                        |

ND : Not detectable.

The extraction of lipids was carried out using chloroform-methanol (2:1) (Folch, Less and Stanley, 1957, J. Biol. Chem., 226: 497). Gas-liquid chromatographic analysis of the fatty acids was carried out after preparation of methyl esters and comparing the retention times of reference standards.

Significant changes were observed in the culture grown under high and low phosphate conditions of growth. The percentage of C₁₄ fatty acids was found to be considerably lower in high phosphate as compared to low phosphate grown cultures (Table I). However, C₁₆, C₁₆:1, C₁₈, C₁₈:2 and C₁₈:3 were in greater percentage in high phosphate grown cells, The percentage of C₁₈:1 fatty acids however, did not register much change.

The alterations in the fatty acid composition along with changes in phospholipids under low and high phosphate conditions further suggests the influence of phosphate at the membrane and hence at the permeability level.

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Rapid degradation of FAD following lysis of *Neurospora crassa* cells: consequences for evaluation of flavin composition in vivo

In an attempt to judge the possible participation of free flavins in blue light photoreceotion processes in *Neurospora*, we determined size and composition of intracellular flavin pools. To minimize artificial liberation of in vivo protein-bound (or associated ) flavins during handling we worked with the cell wall-less mutant 'slime' (FGSC #1118) which can be easily lysed by osmotic shock.

Cells were cultivated in a gyratory shaker (100 rpm) in darkness at 30° C in 250 ml Erlenmeyer flasks containing 50 ml Vogel's minimal medium (Vogel 1956 Microbial. Genet. Bull. 13: 42-43) with 10% sorbitol and 2% sucrose. Under these conditions maximal cell number is reached after 60 h; thus from the third day on, cultures are in the stationary phase. Cultures 2 to 9 d old were harvested (centrifugation 10 min/190 g) and lysed with 4 to 8 ml cold double-distilled water. After centrifugation (Beckman Spinco L 50, 120 min/100,000 g), 2-4 ml of the resulting supernatant were run overnight at 5° C on a Sephadex G-50 medium column (1.55 cm x 90 cm length) in 50 mM KH₂PO₄ buffer at pH 7.0; fraction size was 1.8 ml. The protein content was monitored as A₂₈₀ nm and also tested by the Lowry method. The elution profile of flavins was traced by measuring fluorescent emission at 525 nm upon excitation at 466 nm. Fractions containing flavoprotein or free flavin were...
pooled separately. Analysis of individual flavins in both pools was performed by phenol-extraction (modified after Yagi, 1962, in Meth. Biochem. Anal. Vol. X, Glick ed.: 319-356). After saturation of samples with ammonium sulfate, flavins were extracted twice with phenol, then re-extracted into a small volume of water and separated by TLC on Merck silica gel H type 60 with 135 mM Na2HPO4 as solvent. Flavin concentrations were determined after alkaline photolysis (Yagi 1962) as lumiflavin fluorescence.

From cell lysates of a cell culture of slime, bound and free flavins were separated by Sephadex G-50 filtration. A representative fractionation pattern is shown where bound flavins appeared in fractions no. 21-29 and free flavins in fractions no. 58-70 (Fig. 1). The fluorescence of fractions 21-29 coincided with the protein peak and with the major absorbance maximum at 280 nm. A minor protein peak (peptides?) was observed in fractions 53-56 which appeared before the peak of free flavins; the latter was free of protein. The absorbance at 280 nm in fractions 64-72 is likely due to nonproteinaceous components since there could be no more than 5 µg/ml protein in these fractions as determined by the Lowry method.

Figure 1.-- 100 ml of 2 d old culture were harvested, lysed osmotically in 4 ml of ice-cold double-distilled water and centrifuged (2 h/100,000 g). 2 ml of the resulting supernatant (6.5 mg of protein) were used for separation of free and bound flavins of Sephadex G-50. Fraction size: 1.8 ml; o-o protein content (Lowry method); x-x flavin fluorescence at 525 nm.

TLC analysis of free flavins and flavoprotein fractions after gel filtration of lysates of 2 to 9 d cultures revealed high concentrations of flavin mononucleotide (FMN) and riboflavin (RF) in the free flavin fraction (60 to 40% and 25 to 55% of total flavin present in the fraction, respectively) and about 10% flavin adenine dinucleotide (FAD). In the bound flavin fraction, 11-28% FAD was detected and around 55% and 25% of FMN and RF respectively. 1.0 to 3.3 nmoles of total flavin were put onto the gel.

The unexpectedly low concentration of free FAD in both fractions could have been due to degradation during processing. Adding exogenous FAD to a cell lysate of a 5 d culture and exposing this mixture to our experimental conditions showed fast degradation of the added FAD. Conditions were: a) leaving the mixture for maximally 5 s at 80° C before heating to 80° C, this resulted in a decrease in the FAD content from 89% of total flavin to 53%; b) incubation of mixtures for 30 min at 5° C before heating, this resulted in a decrease in the FAD concentration to 28%; c) after gel filtration at
5° C overnight on Sephadex G-50 (i.e. under the same conditions as in the experiments mentioned earlier) only 25% of total flavin in the eluate was detected as FAD; the amount of FMN increased correspondingly (Table I).

**TABLE I**

Degradation of exogenous FAD in a cell lysate

| Sample | Treatment | RF | FMN | FAD | RF | FMN | FAD |
|--------|-----------|----|-----|-----|----|-----|-----|
| Controls: | | 0.8 | 1.7 | 1.0 | 23 | 48 | 29 |
| (1) Lysate | | 0.8 | 1.5 | 39.0 | 2 | 4 | 94 |
| (2) FAD exog. | calculated flavin content of mixture | 1.6 | 3.2 | 40.0 | 4 | 7 | 89 |
| (1) plus (2) 5 sec at RT | | 4.1 | 11.7 | 27.1 | 10 | 27 | 63 |
| (1) plus (2) 10 min at 5° C | | 7.9 | 26.1 | 13.3 | 17 | 55 | 28 |
| (1) plus (2) G-50 at 5° C overnight | | 1.9 | 26.6 | 9.3 | 5 | 70 | 25 |

* These numbers result from summarizing the absolute nmoles of flavins in lysate plus FADexog. (=44.8 nmoles), 1.6 nmoles of which are RF, 3.2 and 40.0 FMN and FAD, respectively.

250 ml 5 d old culture were lysed in 8 ml double-distilled water, pottered by band and centrifuged (20 min/50,000 gf). 2 ml portions of the supernatant were mixed in the cold with 700 µl TLC-purified FAD and treated with conditions a) b) or c) described in the text. Controls: 1) Flavins extracted from 2 ml supernatant with 18 ml of hot water and 2) 700 µl TLC-purified FAD made up to 20 ml with hot water and taken through the entire phenol-extraction procedure.

**TABLE II**

Heat-lability of FAD-degradation

| Flavin content in nmoles/sample | RF | FMN | FU* | FAD | total |
|---------------------------------|----|-----|-----|-----|-------|
| Control: FAD added | 0.2 | 1.0 | 6.0 | 17.3 | 24.5 |
| Heat treated sample (15 min at 75°C) | 0.5 | 1.7 | 1.0 | 12.9 | 16.1 |
| Sample without heat-treatment | 1.9 | 8.0 | 1.1 | 6.4 | 17.4 |

*unidentified flavin component of commercial FAD-preparation

100 ml of 4 d old culture were divided, harvested and the cells lysed with 20 ml double-distilled water for 15 min at room temperature or at 75° C. The heat-treated sample was cooled to 23° C, 500 µl of unpurified FAD, added to each sample and to a third vial containing 25 ml water. After 15 min at 23° C samples and control were heated for 15 min at 75° C. After cooling and centrifugation of the crude lysates for 20 min/50,000 g, flavins were extracted from the supernatants and the control by the phenol method and separated. The difference of total flavins in the FAD control compared with the heat treated and untreated samples is probably due to loss of flavins during pelleting of membranes and organelles after incubation.
The rapid degradation of FAD in the cell lysate has not been shown to be heat-labile and is likely to be enzyme-catalysed (Table II). These results can be explained by phosphatase(s) splitting off the adenosine group of FAD. The enzyme(s) is reacting fast even at low temperature and within the shortest possible handling time of a few seconds or minutes. Enzyme activities of this kind must be taken into account before making statements on the composition of intracellular flavin pools in Neurospora or other organisms. Data obtained after standard extraction procedures can be perturbed by enzymatic degradation of FAD and/or FMN after decompartmentation following lysis of the cells and may not represent in vivo conditions. We are investigating the possibilities of specific inhibition of the FAD degrading reaction(s).

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