Trace Metal Requirements and some Enzyme Systems in a Riboflavin-requiring Mutant of *Neurospora crassa*

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SUMMARY: A mutant of *Neurospora crassa* is shown to have an absolute requirement for riboflavin when grown at 30°, but this requirement is less stringent when the organism is grown at 25°. The Fe, Cu, Zn, Mn and Mo requirements of the mutant, grown at either temperature, are similar to those of the wild type (146) so that it seems unlikely that these metals are involved in the biosynthesis of riboflavin. A study of enzyme patterns in the mutant, grown at 30° and given optimal or deficient concentrations of riboflavin, demonstrated alternative pathways of electron transfer in the fungus. When riboflavin is deficient, the iron enzymes are markedly increased and oxygen is probably the main terminal acceptor of the electrons. At optimal concentrations of riboflavin, the flavoprotein enzymes are produced and nitrate and nitrite reductases are active so that nitrate can act as a terminal acceptor. Iron deficiency is readily produced in the mutant when riboflavin is deficient because of the increased activity of iron enzymes; a molybdenum requirement is greater at optimal riboflavin concentrations because of the enhanced production of molybdo-flavoproteins.

Mitchell & Houlahan (1946) showed that in a temperature-sensitive mutant of *Neurospora crassa* a riboflavin requirement shown during growth at 30° was eliminated by growing the fungus at 25°. In the present investigation the trace metal requirements of this mutant grown at the two temperatures were determined as well as some of the enzyme changes which occurred when the fungus was grown at 30° with optimum or deficient concentrations of riboflavin.

METHODS

Mutant. The riboflavin-requiring mutant of *Neurospora crassa* which is temperature-sensitive was kindly supplied by Dr A. Mitchell, California Institute of Technology, U.S.A.

Culture medium. The mutant was grown in the following medium: sucrose, 20 g.; NH_4NO_3 , 20 g.; Na tartrate, 1 g.; KH_2PO_4 , 3 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; NaCl, 0.1 g.; CaCl_2 , 0.1 g.; biotin, 5 μg .; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 9.6×10^{-4} g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 8.8×10^{-3} g.; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 2.7×10^{-4} g.; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 7.2×10^{-5} g.; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 1.2×10^{-5} g.; distilled water, 1 litre.

Purification of culture media. The methods used to remove trace metals from the macronutrients were described elsewhere (Nicholas, 1952). The micronutrient elements, Fe, Cu, Zn, Mn and Mo were as 'spectroscopically pure' compounds supplied by Johnson & Matthey (Hatton Garden, London, E.C. 1). Biotin (25 μg .) dispensed in phosphate buffer (pH 7) was supplied in ampoules by the Ashe Laboratories (Leatherhead, Surrey). Riboflavin dispensed in glass-distilled water in an amber bottle was stored in the dark at 0°. The culture

medium (100 ml.) was dispensed in 500 ml. Erlenmeyer flasks, and after sterilizing at 10 lb./sq.in. for 15 min. and cooling, these were inoculated with a mycelial suspension of the mutant in glass-distilled water. Before inoculating and incubating the flasks at 30°, riboflavin was added aseptically (25 µg./100 ml.) when optimal growth of the mutant was required. The organism was grown for 4 days at either temperature

Preparation of cell-free extracts. The mycelial mats collected in a Büchner funnel were washed thoroughly with glass-distilled water and were frozen for 3 hr. at -17°. They were then homogenized in three times their weight of cold 0.1 M-phosphate buffer (pH 7.5) with a pestle and mortar and then in a Ten Broeck glass macerator at 0°. The homogenate was centrifuged at 2000 *g* for 10 min. at 4°. Tris-(hydroxymethyl)-aminomethane buffer 0.1 M (pH 7.5) was used to extract the 'phosphate' enzymes. The homogenates were dialysed for 12 hr. against the same buffers in which they had been prepared.

Cofactors and other compounds. Diphosphopyridine nucleotide (DPN) of 50% purity was prepared from baker's yeast by the method of Kornberg (private communication). Triphosphopyridine nucleotide (TPN) 95% purity and cytochrome *c* were obtained from Sigma Chemical Company, U.S.A., and riboflavin-5-phosphate (FMN) from Nutritional Biochemicals Corporation, U.S.A. Reduced DPN was prepared enzymically by the alcohol dehydrogenase method of Pullman, Colowick & Kaplan (1954) and reduced TPN by using the isocitric dehydrogenase enzyme from acetone powder of pig heart. Boiled pig heart extract, centrifuged at 18,000 *g* for 20 min., at 4° was used as a source of flavine adenine dinucleotide (FAD).

Enzymes assayed

Catalase activity was measured at 37° by the perborate method of Feinstein (1949). The reaction mixture contained 8 ml. 1.5% (w/v) NaBO₃·4H₂O (pH 6.8); 1.5 ml. 0.1 M-phosphate buffer (pH 6.8); 0.1 ml. enzyme extract; and was incubated at 37° for 5 min. Then 10 ml. 2 N-H₂SO₄ was added and the solution titrated with 0.05 N-KMnO₄. The values are expressed as mmole perborate degraded in 5 min./mg. protein.

Peroxidase was determined by the spectrophotometric method of Smith, Robinson & Stotz (1949). The reaction mixture contained 1.8 ml. of McIlvaine's buffer (pH 6.0); 1.0 ml. 10⁻³ M-reduced 2:6-dichloroindophenol; 0.5 ml. 0.1 M-H₂O₂; 0.1 ml. enzyme extract. The oxidation of the dye was followed at 625 mµ. at 15 sec. intervals. The unit of activity is the change in log *I*₀/*I* of 0.001/min. calculated between 15 and 75 sec./mg. protein.

Cytochrome oxidase was measured in homogenates of mycelial felts by the method of Smith & Stotz (1949). The reaction solution contained 1.5 ml. 0.2 M-phosphate (pH 6.8); 1.0 ml. 10⁻³ M-reduced 2:6-dichloroindophenol; 0.5 ml. 1 × 10⁻⁴ M-cytochrome *c*; 0.2 ml. enzyme extract. The units of activity corrected for endogenous rate, are similar to those for peroxidase.

TPN-cytochrome c reductase was measured at 551 mµ. The reaction mixture contained: 2.5 ml. 0.1 M-phosphate buffer (pH 7.5); 0.5 ml. oxidized cytochrome *c*, 3 × 10⁻⁴ M; 0.1 ml. TPNH (2 µmole/ml.) 0.1 ml. enzyme extract.

DPNH and TPNH oxidases ('diaphorase' systems) were measured by following dye reduction at 625 m μ . The reaction mixture used was as follows: 2.5 ml. 0.1 M-phosphate (pH 7.5); 0.8 ml. 1×10^{-3} M-2,6-dichloroindophenol; 0.1 ml. 0.1 M-KCN; 0.1 ml. DPNH (10 mg./ml.) or TPNH (2 μ mole/ml.); 0.1 ml. enzyme extract.

Nitrate reductase was measured by the method of Nicholas & Nason (1954). The test procedure consisted of adding 0.1 ml. enzyme extract to a solution containing 0.1 ml. 0.1 M-KNO₃; 0.04 ml. 10^{-3} M-FMN; 0.02 ml. 10^{-3} M-KCN; 0.04 ml. 2×10^{-3} M-TPNH and 0.80 ml. 0.1 M-phosphate (pH 7.5). After 10 min. incubation at 25°, 3.5 ml. water and 0.5 ml. 1 % (w/v) sulphanilamide reagent and 0.5 ml. 0.001 % (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride naphthylethylenediamine reagent were added. After 10 min. the test solutions were read on the Spekker absorptiometer at 540 m μ . Control tests without TPNH were used to correct for the turbidity of the enzyme. Units of activity are expressed as m μ mole NO₂ formed/mg. protein in 10 min.

Nitrite reductase was determined by a modification of the method described by Nason & Zucker (1956). The test consisted of adding 0.1 ml. enzyme to a solution containing 0.1 ml. 10^{-3} M-KNO₂; 0.05 ml. boiled pig heart extract; 0.05 ml. DPNH (10 mg./ml.) and 0.2 ml. 0.1 M-phosphate buffer (pH 7.5). After 10 min. incubation at 25° the colour was developed and determined as in the nitrate reductase assay.

The following enzymes were examined in 0.1 M-*tris*-(hydroxymethyl)-aminomethane (pH 7.5) extracts of the mycelial felts.

Alkaline phosphatase was estimated by the method of Bessey, Lowry & Brock (1946) in which the breakdown of *p*-nitrophenylphosphate to *p*-nitrophenol was measured. The enzyme activity is expressed as μ mole P released in 30 min./mg. protein.

Hexokinase was measured by the method of Crane & Sols (1953). To 5 ml. solution containing ATP, MgCl₂ and bromothymol blue at 2.5×10^{-3} M; 5×10^{-3} M and 0.0003 % (w/v) respectively is added 0.8 ml. 5 % glucose; 0.2 ml. enzyme extract and the solution adjusted to pH 7.4. The control cell contained water instead of the enzyme. The change in colour was measured at 5 min. intervals for 30 min. at 620 m μ . by means of a spectrophotometer. The enzyme activity is expressed as the change in log I_0/I of 0.001 calculated between 5 and 25 min./mg. protein.

Phosphorylase was measured by the method of Whelan (1955). The reaction mixture contained 0.2 ml. 5 % (w/v) soluble starch; 0.5 ml. 0.5 M-citric acid-NaOH buffer (pH 6.0); 0.1 ml. enzyme extract; 1.7 ml. water equilibrated at 35° for 10 min. and then 1 ml. 0.1 M-glucose-1-phosphate added. The reaction was stopped after 10 min. by adding 5 ml. 5 % (w/v) trichloroacetic acid and after centrifuging at 3000 g for 10 min. a sample was taken for P determination. Control tubes were incubated with acid added at the beginning of the reaction. The activity is expressed in μ mole P formed/mg. protein.

Phosphoglucomutase was determined by the method of Najjar (1955). The reaction mixture contained 0.1 ml. 0.1 M-MgSO₄; 0.1 ml. 0.1 M-glucose-1-phosphate and 0.1 ml. 0.1 M-cysteine hydrochloride (pH 7.5) freshly prepared, which

was equilibrated at 30° before adding 0.2 ml. enzyme extract. The solution was incubated for 10 min. and then 1 ml. 5 N-H₂SO₄ added and the volume made to 5 ml. with distilled water. The reaction tubes were put in a boiling water bath to hydrolyse the remaining glucose-1-P. The control tubes were treated with H₂SO₄ as above at zero time. P was determined in 1 ml. of the reaction mixture. The activity is expressed in μ mole P/mg. protein or μ mole glucose-6-P formed/mg. protein.

Protein was determined by the biuret method of Robinson & Hogden (1940).

RESULTS

Riboflavin requirements

Fig. 1 shows the effect of riboflavin on the growth of the mutant. At 30° there was no growth unless riboflavin was supplied; at 25° appreciable growth occurred without added riboflavin. Maximum yield of the organism was however obtained when the fungus was grown at 30°.

Trace metals in relation to growth

A comparison between the trace metal requirements of *Neurospora crassa* wild type 146 and the riboflavin-requiring mutant grown at 25 and 30° is made in Fig. 2. Irrespective of temperature, Fe, Zn, Cu, Mo and Mn were all required for optimal growth of the mutant. The dry-weight yields of the mutant were similar to those of the wild type 146 when both were grown at 30°, but these were greater than the weights obtained when the mutant was grown at 25°. The micronutrient requirements for optimal growth of the wild type 146 and of the mutant grown at 25° or 30° were similar, being as follows (μ g./100 ml. medium): Fe, 15; Zn, 25; Mo, 1; Cu, 2; and Mn, 2.

Enzymes

Enzyme assays were made in extracts of the mutant grown at 30° at optimal and deficiency concentrations of riboflavin (50 % decrease of weight of fungus). The results for the iron enzymes are given in Table 1. Riboflavin deficiency resulted in a significant increase in peroxidase and cytochrome oxidase. Cytochrome *c* reductase, which has flavin adenine dinucleotide in the prosthetic group, was markedly less in amount when riboflavin was limiting. Catalase was only slightly depressed.

The results for the flavoprotein enzymes are listed in Table 2. The four flavoprotein enzyme systems were less active in riboflavin-deficient mycelium. The nitrate and nitrite reductase enzymes were more markedly decreased in amount than were the diaphorase systems, so that the riboflavin available appeared to be preferentially utilized in the diaphorase systems. The addition of boiled pig heart extract, a source of FAD, restored the activity of these enzymes (except nitrate reductase), almost to the level of controls, in extracts of deficient felts.

The results of assays for four phosphate enzymes are given in Table 3. Hexokinase activity was about 30 % higher in riboflavin deficient mycelium. The other three enzymes appear to be unaffected by riboflavin deficiency.

Fig. 1. Effect of additions of riboflavin on growth of 'riboflavin-requiring' mutant of *Neurospora crassa*. ○—○ growth at 25°; ×—×, growth at 30°.

Fig. 2. Effect of trace metals on growth of wild type (146) and 'riboflavin requiring' mutant of *Neurospora crassa*. ○—○, wild type at 30°; Δ—Δ, mutant at 25°; ×—×, mutant at 30°.

Table 1. *Effect of riboflavin deficiency on the iron enzymes in the mycelium of Neurospora crassa mutant grown at 30°*

Enzyme	Expt. no.	Normal mycelia	Riboflavin-deficient mycelia
		Units of enzyme activity/mg. protein	
Catalase	1	1160	812, 70 % of normal
	2	1475	1244, 85 % of normal
	3	1494	1256, 84 % of normal
Peroxidase	1	38	91, 240 % of normal
	2	54	83, 154 % of normal
	3	150	409, 270 % of normal
Cytochrome <i>c</i> oxidase	1	300	464, 155 % of normal
	2	117	148, 127 % of normal
	3	663	804, 121 % of normal
Cytochrome <i>c</i> reductase	1	275	92, 33 % of normal
	2	145	38, 26 % of normal
	3	180	60, 33 % of normal

Table 2. *Effect of riboflavin deficiency on flavoprotein enzymes in the mycelium of Neurospora crassa mutant grown at 30°*

(Mean of 4 experiments)

Enzyme	A Normal	B Riboflavin-deficient mycelia	FAD added <i>in vitro</i> to extracts of riboflavin deficient mycelia (% of A)	
	Units of enzyme activity/mg. protein		B as % of A	
DPNH diaphorase	975	485	50	98
TPNH diaphorase	783	242	31	79
Nitrate reductase	19.4	2.0	10	10
Nitrite reductase	15	2.5	16	94

Table 3. *Effect of riboflavin deficiency on 'phosphate' enzymes in the mycelium of Neurospora crassa mutant grown at 30°*

Enzyme from	Phosphatase	Hexokinase	Phosphorylase	Phosphoglucumutase
	Units of enzyme/mg. protein			
Control mycelium	1.65	131	1.09	2.14
Riboflavin-deficient mycelium	1.50	180	1.08	1.83

DISCUSSION

A temperature-sensitive mutant of *Neurospora crassa* is shown to have an absolute requirement for riboflavin when grown at 30°, but this is markedly less exacting when grown at 25°. The reason for the different requirement for the vitamin at the two temperatures cannot be explained in terms of a decrease in amount of flavoprotein enzymes in the fungus grown at 25°. In fact there

is no difference between the activity of the flavoprotein enzymes in the mutant grown at the two temperatures so that riboflavin must be synthesized from its precursors in the fungus at 25° (unpublished result).

There is a similar requirement for Fe, Zn, Cu, Mn and Mo in the wild type 146 and for the mutant grown at 25° or 30°. It seems unlikely therefore that these metals are involved in the biosynthesis of riboflavin in the mutant.

The results of enzyme assays of the mutant grown at 30° with optimal and deficient concentrations of riboflavin demonstrate alternative mechanisms for electron transport in the mould. When riboflavin is deficient two iron enzymes (peroxidase and cytochrome oxidase) are produced in large amounts and oxygen is probably the terminal electron acceptor because the flavoprotein enzymes, nitrate and nitrite reductases, are considerably diminished in quantity. The TPNH and DPNH diaphorase systems and cytochrome *c* reductase, which are flavin dependent, are also much decreased in amount when riboflavin is deficient. Iron deficiency is more readily produced in riboflavin-deficient mycelium, when the iron enzymes are produced in quantity, and the Mo requirement is greater when the riboflavin concentration is optimal, the flavoprotein enzymes then being very active. Thus the electron transfer mechanism of this organism involves an iron system when riboflavin is deficient and a molybdoflavoprotein system when the riboflavin concentration is optimal. The effects of Fe and Mo deficiencies on electron transfer in the fungus are similar to those reported for *Pseudomonas fluorescens* by Lenhoff, Nicholas & Kaplan (1956).

The effect of FAD (boiled pig heart) or FMN in reactivating the DPNH and TPNH diaphorase systems and nitrite reductase in extracts of riboflavin-deficient mycelium shows that their apoenzymes are unaffected by riboflavin deficiency. A shortage of riboflavin did not affect the flavoprotein enzymes to the same degree; thus nitrate and nitrite reductases are more markedly decreased in amount than are the DPNH or TPNH diaphorase systems. This may be explained in terms of a greater affinity of the apoenzyme of some flavoproteins for the flavin nucleotides available; the ones having the greatest affinity would retain their activity longest.

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