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Changes in Enzymes Regulating Isocitrate Breakdown in Neurospora crassa

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With 2 Figures in the Text

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Microorganisms growing on acetate as a carbon source shunt part of the Krebs cycle by a "glyoxylate by-pass" which can replenish the carboxylic acids drained away during growth (KORNBERG and ELSDEN 1961). The glyoxylate shunt is initiated in the presence of acetate by induction of the isocitrate lyase cleaving isocitrate into glyoxylate and succinate (SMITH and GUNSALUS 1955). An increase of isocitrate lyase activity during growth on an acetate containing medium has been shown in *Neurospora* by TURIAN et al. (1962). It was of interest to determine whether or not the oxidative breakdown of isocitrate through the Krebs cycle was correlated with the stimulation of isocitrate lyase.

Material and Methods

From a conidial inoculum, *Neurospora crassa* (strain Lindegren +) was grown for four days at 25° C in separate flasks, shaken at a rate of 110 strokes per minute. The Westergaard-Mitchell liquid medium (WESTERGAARD and MITCHELL 1947) was altered with respect to sucrose concentration and, for certain experiments, was supplemented with various concentrations of sodium acetate. After filtration and pressing off excess water, the cultures were weighed and transferred to the cold room. They were ground in mortars with acid cleaned sea sand in a 0.2 M phosphate buffer (pH 6.0) containing 0.5 mM cystein, 1 mM EDTA[1] and traces of an anionic detergent (polyethoxyether-alcoyl-phenol). The homogenate (1 to 4 final dilution in buffer) was spun down at 16,000 × g for 20 min in a Servall SS-1 centrifuge. Aliquots of the supernatant were saved for enzyme and protein determinations.

As a rule, all enzymes were assayed in a small volume of stock reagent; necessary additions were made with Lang-Levy constriction pipettes. Unless otherwise stated, the reaction was started by addition of substrate and its course was followed in a Beckman DU spectrophotometer.

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[1] Abbreviations: AMP = adenosine-5′-phosphate; EDTA = ethylene-diamine tetraacetate; NAD = nicotinamide-adenine dinucleotide; NADP = nicotinamide-adenine dinucleotide phosphate; TRIS = tris-(hydroxymethyl)-aminomethane.

Isocitrate lyase. Method adapted from DIXON and KORNBERG (1959). Reagent: 0.05 M imidazole buffer (pH 7.2); 3.9 mM $MgCl_2$; 3.9 mM cystein; 2.6 mM phenylhydrazine; 7.7 mM D,L-isocitrate. The maximum velocity of appearance of glyoxylate-phenylhydrazone was recorded at 324 mμ after 10 min allowance for lag.

NADP isocitrate dehydrogenase. 0.1 M TRIS buffer (pH 8.1); 0.05% bovine serum albumin; 0.2 mM $MnCl_2$; 0.5 mM NADP; 7.1 mM D,L-isocitrate.

NAD isocitrate dehydrogenase. 0.1 M TRIS buffer (pH 8.1); 0.05% bovine serum albumin; 0.2 mM $MnCl_2$; 0.2 mM AMP (not standardized enzymatically); 5 mM NAD; 8.0 mM D,L-isocitrate.

NADP glutamic dehydrogenase (BARRATT 1963).

NADase was measured in an incubation medium adapted from KAPLAN, COLOWICK and NASON (1951). To 1 ml of 0.1 M phosphate buffer (pH 6.45), NAD was added with a micropipette to a final concentration of 3.3 mM. The reaction was started by addition of homogenate. At intervals, a sample of 1.11 μl was transferred from the incubation mixture to fluorometric tubes containing 1 ml of 6 N NaOH. These tubes were heated for 10 min at 60° C in a water bath, then brought back to room temperature. The resulting fluorescence of NAD (KAPLAN, COLOWICK and BARNES 1951) was read in a Farrand fluorometer Model A-2 (primary beam: 340 mμ; secondary beam: 435 mμ). The amounts of NAD destroyed by two different concentrations of homogenate during various incubation times were compared to known concentrations of NAD, previously standardized as a cyanide complex at pH 11, using $\varepsilon_{327} = 5.9 \cdot 10^3$. Blanks, standards and samples were run simultaneously.

NADP was standardized in the following reagent: 0.1 M TRIS buffer (pH 8.1); 0.2 mM $MnCl_2$; pig heart isocitrate dehydrogenase (Boehringer u. Söhne) 20 μg. Addition of 7.3 mM D,L-isocitrate started the reaction. Isocitrate was standardized in the same basic reagent, made 0.5 mM in NADP.

Protein determination was made according to LOWRY et al. (1951) in 1 ml of reagent, using bovine serum albumin as a standard. The color was read at 750 mμ.

Respiration was measured by the conventional Warburg "direct" method (UMBREIT et al. 1957).

Results

A. Optimal conditions for enzyme determinations

Cofactor requirements for enzymatic activities are reported in Table 1.

Isocitrate lyase from *Neurospora* has an absolute requirement for sulfhydryl group, provided here by cystein. Mg^{2+} greatly enhances activity, although not essential.

Isocitrate oxidation in presence of NADP proceeds even without addition of Mn^{2+}. Catalytic concentration of this metal increases significantly the activity, although maximum velocity is reached at somewhat higher concentrations. Isocitrate oxidation in presence of NAD shows an absolute requirement for Mn^{2+}. AMP is necessary for full activity; this adenylate is already known as a cofactor of NAD isocitrate dehydrogenase in yeast (KORNBERG and PRICER 1951) and in *Aspergillus* (RAMAKRISHNAN and MARTIN 1955).

KAPLAN, COLOWICK and NASON (1951) do not report any cofactor requirement for NADase from *Neurospora*; therefore, no further in-

Table 1. *Effect of cofactor concentration on activity of isocitrate-catalyzing enzymes*

Isocitrate lyase[1]				NADP isocitrate[2] dehydrogenase		NAD isocitrate[3] dehydrogenase			
Cystein	Activity	$MgCl_2$	Activity	$MnCl_2$	Activity	$MnCl_2$	Activity	AMP	Activity
0.0	0.0	0.0	0.12	0.00	3.6	0.00	0.0	0.00	8.7
0.39	2.48	0.39	1.18	0.002	14.6	0.02	74.5	0.02	38.8
0.50	24.5	0.50	53.0	0.011	53.7	0.04	90.4	0.04	56.4
3.9	69.6	3.9	70.2	0.022	63.0	0.079	96.2	0.10	67.5
8.0	69.0	8.0	69.7	0.195	71.6	0.154	100.0	0.20	69.2
				0.353	71.6	0.478	99.0		

[1] Homogenates from culture 0.014 M sucrose — 0.110 M acetate. Analytical conditions: 0.05 M imidazole buffer pH 6.9; 4.25 mM phenylhydrazine; 4.12 mM D,L-isocitrate. Effect of cystein tested in presence of 3.9 mM $MgCl_2$; effect of $MgCl_2$ tested in presence of 3.9 mM cystein. 24.1 μg protein in cuvette.

[2] Homogenate from culture 0.007 M sucrose — 0.074 M acetate; 2.49 μg protein in cuvette.

[3] Homogenate from culture 0.147 M acetate. Effect of $MnCl_2$ tested in presence of 0.2 mM AMP; effect of AMP tested in presence of 0.2 mM $MnCl_2$; 1.85 μg protein in cuvette.

Concentrations in mM; activities in mμmoles/h.

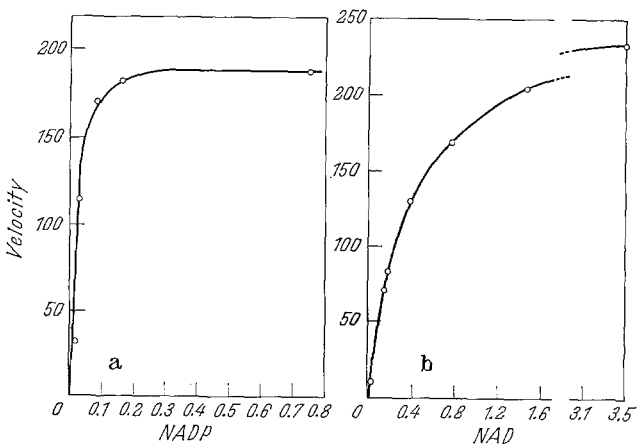

Fig. 1a and b. Effect of coenzyme concentration on velocity of isocitrate-oxidizingenzymes. a Effect of NADP concentration (in mM) on velocity (in mμmoles/h) of NADP isocitrate dehydrogenase from 0.014 M sucrose—0.110 M acetate culture (7.50 μg protein in cuvette); b Effect of NAD concentration (in mM) on velocity (in mμmoles/h) of NAD isocitrate dehydrogenase from 0.147 M acetate culture (24.0 μg protein in cuvette)

vestigation was carried out in this direction. BARRATT's (1963) optimal conditions for NADP glutamic dehydrogenase in *Neurospora* were adopted without modification.

Coenzyme requirements are plotted in Fig. 1. Affinity for NADP is high; in contrast, the velocity of NAD reduction reaches its maximum value for unusually high concentrations of NAD, even though both

cofactors are at their optimal levels. This result is reproducible and is not an artefact due to NAD split during the reaction: the rates remain constant with time and the homogenates used for these assays are devoid from NADase activity. Occurrence of two distinct dehydrogenases is suggested by: a) differences in cofactor requirements; b) differences in affinities for substrate (Fig. 2 left); c) summation of activities obtained by mixing both coenzymes (Table 2); d) differential reactivity of NAD- and NADP-dependent oxidation rates to sucrose depletion in the growth medium (Table 4). However, optimal pH's are similar (Fig. 2 right). Positive identification of an NAD isocitrate dehydrogenase in *Neurospora* has been made by SANWAL et al. (1964).

Table 2. *Summation of isocitrate oxidation rates in presence of NADP and/or NAD*
Homogenate from culture 0.007 M sucrose

	Coenzyme added (mM)	Specific activity (μmoles/h/mg protein)
NADP	0.495	9.0
NAD	4.22	13.9
NADP + NAD	0.487 + 4.16	24.5

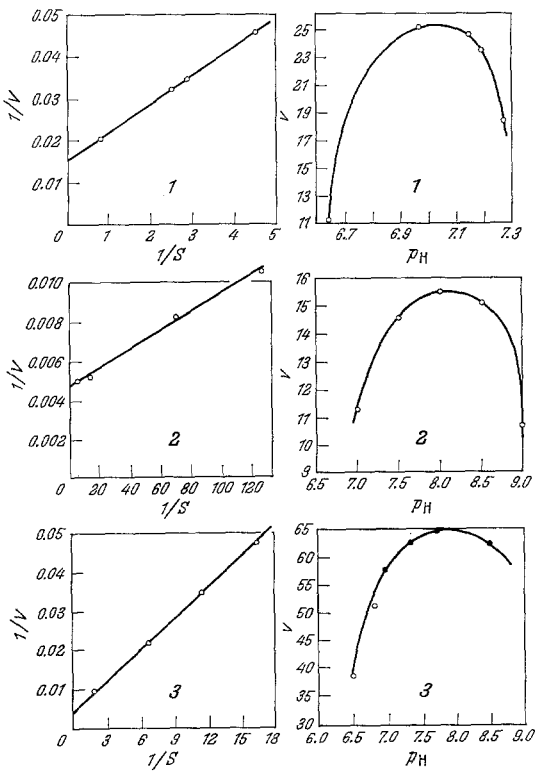

Fig. 2. Kinetic data on isocitrate-metabolizing enzymes. Left: Effect of D,L-isocitrate concentration (1/S in mM) on velocity (1/v in mμmole/h) of: *1* isocitrate lyase from 0.014 M sucrose—0.110 M acetate culture (28.0 μg protein in cuvette); *2* NADP isocitrate dehydrogenase from 0.014 M sucrose—0.110 M acetate culture (10.1 μg protein in cuvette); *3* NAD isocitrate dehydrogenase from 0.007 M sucrose—0.147 M acetate culture (8.84 μg protein in cuvette). Right: Effect of pH on velocity (v in mμmoles/h) of: *1* isocitrate lyase from 0.014 M sucrose—0.110 M acetate culture (11.0 μg protein in cuvette); *2* NADP isocitrate dehydrogenase from 0.014 M sucrose—0.110 M acetate culture (7.06 μg protein in cuvette); *3* NAD isocitrate dehydrogenase from 0.147 M acetate culture (protein content not recorded); open circles 0.1 M imidazole buffer

B. Effect of nutritional conditions

Substitution of sucrose by acetate in the growth medium increases the specific activities of isocitrate lyase and of NADP isocitrate dehydrogenase;

NADP glutamic dehydrogenase is not affected (Table 3). In this type of experiment, the increase of the ratio acetate/sucrose results in a higher concentration of nutrient and in a lower number of

Table 3. *Effect of substitution of sucrose by acetate on isocitrate lyase, NADP isocitrate dehydrogenase, NADP glutamic dehydrogenase and respiration*

Nutrient source (M)	Isocitrate lyase (μmoles/h/mg protein)	NADP isocitrate dehydrogenase (μmoles/h/mg protein)	NADP glutamic dehydrogenase (μmoles/h/mg protein)	Q_{O_2}
Sucrose 0.058	0.442	4.12	75.6	8.68
Sucrose 0.054 Acetate 0.015	0.507	8.18	74.5	9.58
Sucrose 0.044 Acetate 0.037	0.698	11.1	74.2	15.4
Sucrose 0.029 Acetate 0.074	0.894	15.9	—	18.1
Sucrose 0.014 Acetate 0.110	2.38	21.9	75.0	20.3
Sucrose 0.006 Acetate 0.132	5.27	44.2	74.3	25.2
Acetate 0.147	9.57	58.2	77.2	28.7

Table 4
Effect of sucrose concentration on isocitrate-catalyzing enzymes and on NADase

Sucrose (M)	Isocitrate lyase (μmoles/h/mg protein)	NADP isocitrate dehydrogenase (μmoles/h/mg protein)	NAD isocitrate dehydrogenase (μmoles/h/mg protein)	NADase (μmoles/h/mg protein)
0.114	0.369	3.88	8.10	216
0.058	0.431	4.22	8.25	162
0.042	0.548	4.37	8.70	121
0.037	0.576	6.92	10.4	110
0.030	0.672	7.25	10.8	—
0.015	0.780	10.4	11.2	105
0.007	1.06	12.7	12.5	83
0.003	1.32	16.9	13.5	80

carbon atoms. In an attempt to decide whether or not changes in the concentration of nutrient could by themselves produce the observed changes of enzyme activities, *N. crassa* was grown on various concentrations of sucrose and the crude homogenates were tested for enzymatic activities. It appears from the data recorded in Table 4 that sucrose reduces the specific activity of the three enzymes regulating isocitrate breakdown. However, the lowering of the sucrose concentration, even 19-fold below normal (0.058 M) does not raise activities to

the levels reached after growth on acetate. On the other hand, a concentration of sucrose twice as high as normal produces a slight, but reproducible, inhibitory effect on the specific activity of these enzymes.

The role of acetate in stimulating activities of isocitrate lyase, NADP- and NAD isocitrate dehydrogenases appears when *N. crassa* is grown on sucrose media supplemented with two different concentrations of the C_2 compound (Table 5). Activities, already heightened by sucrose depletion from the medium, are further stimulated by acetate.

Table 5
Effect of acetate supplementation on isocitrate-catalyzing enzymes and on NADase

Nutrient source (M)	Isocitrate lyase (μmoles/h/mg protein)	NADP isocitrate dehydrogenase (μmoles/h/mg protein)	NAD isocitrate dehydrogenase (μmoles/h/mg protein)	NADase (μmoles/h/mg protein)
Sucrose 0.058	0.492	3.79	7.77	143
Sucrose 0.058 Acetate 0.074	0.586	7.13	10.8	57.6
Sucrose 0.058 Acetate 0.147	0.619	10.6	15.3	27.8
Sucrose 0.007	1.32	10.5	14.1	12.2
Sucrose 0.007 Acetate 0.074	2.14	30.9	24.1	0.1
Sucrose 0.007 Acetate 0.147	4.51	55.8	37.7	0

Enzymatic cleavage of coenzyme may prevent estimation of NAD-requiring enzyme in cell-free conidial extracts (ZALOKAR and COCHRANE 1956). Evidence for NADP destruction during determination of NADP isocitrate dehydrogenase in homogenates from cultures grown on 0.058 M sucrose is based on the following observations: a) the rate of the reaction slows down after approximately 10 min; b) the reduced coenzyme is not destroyed; c) subsequent addition of NADP, but not of isocitrate, returns oxidation to its original rate. Since the same enzyme splits both NAD and NADP (KAPLAN, COLOWICK, and NASON 1951), experiments were restricted to measures of NADase activities in various conditions of carbon supplementation.

Table 6
Effect of rinsing on NADase activity

Volume of buffer used (ml)	NADase activity (μmoles/h/mg protein)
0	190
200	148
500	114
1270	88

Lowering the sucrose concentration in the growth medium reduces NADase activity (Table 4). A more striking inhibition is produced by

addition of acetate (Table 5). On NADase sucrose and acetate appear to have antagonistic actions.

NADase can be removed from conidia by washing them with buffer (ZALOKAR and COCHRANE 1956). A comparable result was obtained by rinsing the mats of shake cultures with increasing volumes of 0.2 M phosphate buffer (pH 6.0). Despite of the large volume of buffer used, almost 50% of the original amount of enzyme is retained in the washed cells (Table 6); it is therefore assumed that NADase could be active enough to interfere significantly with the *in vivo* activity of NAD isocitrate dehydrogenase.

Discussion

In our vigorously shaken liquid cultures of *N. crassa*, rather uniform growth of short, separate young hyphae is obtained. The ratio of the shorter, conidia-like elements to the somewhat longer hyphae is fairly constant, regardless of the amount or nature of the carbon source. Therefore, in those growth conditions, the observed changes in enzyme activities cannot be correlated with any particular morphological variation.

Our experiments do not demonstrate whether or not the various enzyme activities recorded result from differences in the actual amounts of enzymes. However, summation of activities consistently obtained when a highly active preparation is added to a poorly active homogenate rules out the possibility of any inhibitor's interfering significantly with enzyme measurement. NADP glutamic dehydrogenase, an enzyme unrelated to isocitrate breakdown, is not affected by the various nutritional conditions. Therefore, the correlation in activities between isocitrate-catalyzing enzymes can be regarded as specific and not as the result of some gross change in the enzymatic stock. The progressive increase of Q_{O_2} following the raise of the acetate/sucrose ratio in fresh media (Table 3) indicates that acetate is used along with sucrose; therefore, the values of enzymatic activities cannot be connected with a possible sequential utilization of the carbon sources (diauxie).

The specific activities of the enzymes oxidizing or splitting isocitrate are not correlated either with the total amount of the carbon sources or with a starvation effect, which becomes apparent (reduction of the fresh weight) only for concentrations of sucrose below 0.030 M. It appears from the results that both sucrose and acetate control, with different efficiencies, the specific activities of the enzymes catalyzing isocitrate breakdown. Sucrose (or presumably some metabolite derived from it) inhibits these activities; acetate does not only overcome sucrose inhibition but also produces a marked stimulation. It is concluded that the physiological adaptation of *N. crassa* to utilization of acetate as a carbon source involves an increase of isocitrate breakdown, by oxidation as

well as by cleavage. This accounts for accumulation of both glyoxylate and α-ketoglutarate observed by OWENS (1955) in conidia of *N. sitophila* incubated during 2 hours on acetate.

The nutritional conditions increasing activities of isocitrate metabolizing enzymes have a completely inverse effect on NADase. Since NAD isocitrate dehydrogenase has a very high requirement for NAD, the *in vivo* activity of this enzyme is likely to be limited by NAD availability, which is itself regulated by NADase activity. Sucrose depletion and acetate supplementation inhibit NADase activity, thus leaving more NAD available for operation of NAD isocitrate dehydrogenase. The same process might also regulate the NADP isocitrate dehydrogenase, but with a lower efficiency, owing to the higher affinity of this enzyme for its coenzyme. A large fraction of NADase appears to be surrounding the cells, as showed by its ability to be washed out of the mat. It is therefore difficult to appreciate to what extent the *in vivo* activities of both dehydrogenases are actually limited by enzymatic cleavage of coenzymes. Nevertheless, an alteration of NADase following adaptation to acetate might trigger an additional mechanism resulting in a higher rate of isocitrate oxidation.

No evidence of a switch from the Krebs to the glyoxylate cycle can be found at the level of isocitrate which, in our experimental conditions, does not act as an alternating branching point. Succinate, a known intermediate of the Krebs cycle, is able to inhibit isocitrate lyase synthesis (KORNBERG, COLLINS and BIGLEY 1960) and, consequently, to restrain the glyoxylate shunt. Isocitrate oxidation, promoted by acetate, would be expected to yield higher succinate levels and, therefore, limit isocitrate lyase formation. However, accumulation of α-ketoglutarate in acetate-grown *Neurospora* (TURIAN 1963) suggests the appearance of a block preventing succinate from reaching a level high enough to repress isocitrate lyase formation.

Summary

1. Crude homogenates from *Neurospora crassa* grown on various concentrations of sucrose and acetate were assayed for enzymes regulating isocitrate breakdown.

2. Specific activities of isocitrate lyase, NADP- and NAD isocitrate dehydrogenases were increased by depletion of sucrose or supplementation with acetate.

3. High sucrose concentrations in the growth medium enhanced NADase activity, whereas addition of acetate strongly inhibited this enzyme.

4. It was concluded that the physiological adaptation of *N. crassa* to sucrose depletion or acetate supplementation involves: a) an increase

of isocitrate cleavage into succinate and glyoxylate; b) an acceleration of isocitrate oxidation to α-ketoglutarate.

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References

BARRATT, R. W.: Effect of environmental conditions on the NADP-specific glutamic acid dehydrogenase in *Neurospora crassa*. J. gen. Microbiol. **33**, 33 (1963).

DIXON, G. H., and H. L. KORNBERG: Assay methods for key enzymes of the glyoxylate cycle. Biochem. J. **72**, 3 P (1959).

KAPLAN, N. O., S. P. COLOWICK, and C. C. BARNES: Effect of alkali on diphosphopyridine nucleotide. J. biol. Chem. **191**, 461 (1951).

— — and A. NASON: *Neurospora* diphosphopyridine nucleotidase. J. biol. Chem. **191**, 473 (1951).

KORNBERG, A., and W. E. PRICER: Di- and triphosphopyridine nucleotide isocitric dehydrogenases in yeast. J. biol. Chem. **189**, 123 (1951).

KORNBERG, H. L., J. F. COLLINS, and D. BIGLEY: The influence of growth substrates on metabolic pathways in *Micrococcus denitrificans*. Biochim. biophys. Acta (Amst.) **39**, 9 (1960).

—, and S. R. ELSDEN: The metabolism of 2-Carbon compounds by microorganisms. Advanc. Enzymol. **23**, 401 (1961).

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL: Protein measurement with the Folin phenol reagent. J. biol. Chem. **193**, 265 (1951).

OWENS, R. G.: Metabolism of fungus spores. I. Oxidation and accumulation of organic acids by conidia of *Neurospora sitophila*. Contrib. Boyce Thomson Inst. **18**, 125 (1955).

RAMAKRISHNAN, C. V., and S. M. MARTIN: Isocitric dehydrogenase in *Aspergillus niger*. Arch. Biochem. **55**, 403 (1955).

SANWAL, B. D., M. W. ZINK, and C. S. STACHOW: Nicotinamide adenine dinucleotide-specific isocitrate dehydrogenase. A possible regulatory protein. J. biol. Chem. **239**, 1597 (1964).

SMITH, R. A., and I. C. GUNSALUS: Distribution and formation of isocitritase. Nature (Lond.) **175**, 774 (1955).

TURIAN, G.: Sur le mécanisme de l'induction isocitratasique chez *Allomyces* et *Neurospora*. Path. et Microbiol. (Basel) **26**, 553 (1963).

— J. SEYDOUX et D. VOLKMANN: Activité isocitratasique et type de sporulation chez *Neurospora tetrasperma* et chez *N. sitophila*, souche normale et mutant microconidien. Path. et Microbiol. (Basel) **25**, 737 (1962).

UMBREIT, W. W., R. H. BURRIS, and J. F. STAUFFER: Manometric techniques. Minneapolis: Burgess Publ. 1957.

WESTERGAARD, M., and H. K. MITCHELL: *Neurospora*. V. A synthetic medium favoring sexual reproduction. Amer. J. Bot. **34**, 573 (1947).

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