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Altered Fatty Acid Distribution in Mutants of *Neurospora crassa*

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Morphological mutants of *Neurospora* with decreased levels of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and reduced nicotinamide adenine dinucleotide (NADH) contained only 20% as much of a polyunsaturated fatty acid (linolenic acid) as the wild type in both the phospholipid and neutral lipid fractions. There was an excellent correlation between linolenic acid levels and morphological appearance as a function of total NADPH content, but no correlation with NADH content. The linolenic acid deficiency was balanced by a relative increase in the amounts of the less unsaturated fatty acids (oleic and linoleic acids), but the level of three other fatty acids did not appear to be changed. This accumulation of these two precursors suggests that the NADPH deficiency preferentially affected the final desaturation step, i.e., the conversion of linoleic to linolenic acid. The NADPH needed for this reaction in vivo was probably generated by the pentose phosphate shunt, since mutations affecting the shunt lead to the decreased levels of linolenic acid. It is not clear whether the changes in fatty acid distribution affect the morphogenesis of *Neurospora*, or if these changes are just part of the NADPH-deficiency syndrome.

The primary biochemical deficiency of a morphological mutant of *Neurospora crassa* (col-2) was described (3) as an alteration in glucose-6-phosphate dehydrogenase (EC 1.1.1.49), an enzyme which generates reduced nicotinamide adenine dinucleotide phosphate (NADPH). One pleiotropic effect produced by this mutational alteration was a decrease in the total content of NADPH as compared to that of the wild-type strain (2). The effects produced in turn by a decreased content of this cofactor could be numerous, but since it is generally thought that NADPH is primarily used for the synthesis of fatty acids and for the formation of unsaturated bonds in fatty acids, it was decided to investigate these possibilities first. NADPH is needed in those reactions catalyzed by mixed-function oxidases of the general reaction type: saturated fatty acid + O_2 + NADPH yielding unsaturated fatty acid + nicotinamide adenine dinucleotide phosphate (NADP) + water. This paper reports the effects of an overall NADPH deficiency on the distribution of fatty acids in the phospholipid and neutral lipid fractions of *Neurospora*.

MATERIALS AND METHODS

Cultures. RL3-8A (wild type), col-2, col-3, rg, col-2b$_2$ inosS1, and col-2b$_2$ inosR11 (revertant) have all been described previously (3, 4). Another morphological mutant (balloon) was obtained from the Fungal Genetics Stock Center, Dartmouth College, Hanover, N.H., as well as the nicotinic acid or tryptophan-requiring strain (nt). Double-mutant strains were constructed by the appropriate crosses.

Growth conditions. All cultures were grown as liquid shake cultures in Vogel's minimal medium (10) containing 2% glucose at 23 C, unless otherwise stated. The col-2 nt strain received a supplement of 20 μg of nicotinic acid per ml. The growth and shearing (grinding in a sterile Waring Blendor) procedures were described previously (2). Cultures were rapidly harvested by suction filtration, washed with distilled water, frozen for a few hours, and lyophilized overnight.

Fatty acid analyses. Freshly lyophilized samples of mycelium (about 100 mg) were immediately immersed in 5 ml of methanol at -15 C for short periods of time. Lipids remaining in the mycelium after the initial treatment with methanol were extracted with two successive 5-ml volumes of chloroform-methanol (2:1) in a ground-glass homogenizer. The combined extracts were washed by the method of Folch et al. (8) and dried with magnesium sulfate. The total lipid extracts were separated into neutral lipids and phospholipids on chromatographic columns (18 mm inside diameter) with 15 g of silicic acid as the stationary phase, by the general method of Fillerup and Mead (7), with eluting solvents based on the work of Borgstrom (1). A 5-g amount of fine sand was de-

posited above the silicic acid to minimize distortion of the column during the elution procedure. Lipid samples were applied to the column after equilibrating the stationary phase with chloroform. The neutral lipids were eluted with 75 ml of chloroform and the phospholipids were eluted with 75 ml of methanol.

The eluates were taken to smaller volumes, and the component fatty acids in the lipids were converted to the corresponding methyl esters by acid-catalyzed transesterification. The fatty acid analyses were carried out by using a Loenco model 15B gas chromatograph with a 183-cm (6 ft) column of 20% diethylene glycol succinate on Chromosorb G. The operating temperature was 197 C, with a gas flow of 60 ml/min through a column of 6 mm (¼ inch) diameter. The detector response for the methyl esters of fatty acids was determined with standards of known composition. The peak area was determined by multiplication of peak height by one-half base width. Precautions were taken to minimize fatty acid oxidation during these studies. Solvents were degassed before use with nitrogen, and lipid solutions were kept under an atmosphere of nitrogen whenever this was feasible. In this paper, the absolute amounts of fatty acids are given in micromoles per gram of dry weight (after lyophilization). In previous papers, NADPH was expressed in terms of micromoles per gram of residual dry weight. To compare these two parameters directly, 1 g of lyophilized mycelia will have a residual dry weight, after extraction, of 0.70 to 0.80 g. All cultures were grown in duplicate, unless otherwise stated, and each extract was analyzed for fatty acid composition at two different concentrations of the extract.

RESULTS

Fatty acid variations in phospholipids. The col-2 phospholipids contained only about 20% as much linolenic acid as did the corresponding wild-type fraction (Table 1). The values given for the col-2 cultures were obtained from sheared, 2-day-old cultures, since 8- to 10-day cultures of the slow-growing col-2 often contained even less linolenic acid, perhaps due to oxidation in the older, vacuolated areas of the mycelia. The col-2 strain was previously shown to contain only 40% of the NADPH as the wild type (2). A temperature-sensitive partial revertant strain (col-2b_2 inosS1), when grown at 23 C, had a wild-type level of NADPH but an intermediate level of linolenic acid. When grown at 34 C, this strain had a compact colonial morphology, low level of NADPH, and a low level of linolenic acid. A revertant strain (R11), derived by back mutation at the col-2 locus, had wild-type morphology, normal levels of NADPH, and nearly wild-type levels of linolenic acid. This last observation indicates that the lowered level of linolenic acid was related to the col-2 mutation and not to the genetic background.

Phospholipid extracts of two other morphological mutants (balloon and col-3) were also low in linolenic acid content (Table 2). These two mutants were reported to be deficient in NADPH content (2), owing to mutations affecting glucose-6-phosphate dehydrogenase (9) and 6-phosphogluconic acid dehydrogenase (J. F. Lechner and K. E. Fuscaldo, Bacteriol. Proc., p. 120, 1969), respectively. Another morphological mutant (rg), altered in phosphoglucomutase (4), was similar to the wild type in both NADPH content (2) and linolenic content (Table 2). The normal fatty acid composition of the rg strain indicates that a low growth rate or an altered metabolism due to a morphological mutation did not in itself necessarily lead to a lowered linolenic acid content. Rather, the linolenic deficiency seems to have been specifically related to those mutations (col-2, col-3, balloon) which produced lower levels of NADPH. Further evidence along this line is seen in Table 1, where it is apparent that the wild type, when grown on acetate as sole carbon source at 34 C, produced a lower total content of NADPH, a semicolonial morphology, and lower amounts of linolenic acid.

The correlation of the linolenic acid levels with NADPH levels is stressed above, rather than with NADH levels, as in Fig. 1A and B. The NADPH and NADH levels were reported previously (2) and are plotted here as a range of values, since Chance et al. (5) indicated that these levels may normally show cyclic oscillations about a mean value. Figure 1A shows a graphic relationship between the total content of two different molecular species and is not to be construed as a kinetic plot of any sort.

Another line of evidence suggested that there was a correlation of linolenic acid levels with NADPH levels, but not with NADH content. Table 2 indicates that the col-2 nt strain (requiring either nicotinic acid or tryptophan), when grown with a supplement of nicotinic acid, had low levels of linolenic acid. However, it was found that this strain differed from col-2 in that it contained higher levels of NADH (0.72 to 0.82 μmole/g), but the same levels of NADPH (0.11 to 0.15 μmole/g). Therefore, only the NADPH and linolenic acid levels were correlated.

The lower percentage of linolenic acid in the col-2 phospholipids appeared to be compensated for by the increase in the relative molar amounts of linoleic and oleic acids (Table 1). An increase in the sum of these two fatty acids was shown in all cases (Tables 1 and 2) where the linolenic content was lower than the wild-type level. In fact, at 23 C, the sum of the oleic, linoleic, and linolenic acid content of the phospholipids was a constant 66 to 70% of the total fatty acids in all strains. These findings suggest that the NADPH deficiency in col-2 led to a partial block only in

TABLE 1. *Distribution[a] of fatty acids in phospholipids*

Strain	Growth temp	Morphology	NADPH[b]	Fatty acids in phospholipids[c]	Palmitic	Stearic	Oleic	Linoleic	Linolenic
	C								
RL3-8A	23	Wild type	0.23–0.29	35–45	27.5 ± 1.5 SE[d]	2.0 ± 0.33 SE	7.0 ± 0.75 SE	48.0 ± 0.36 SE	14.1 ± 1.1 SE
RL3-8A	34	Wild type	0.22–0.30	46	24.4	4.5	11.9	45.4	13.5
col-2	23	Colonial	0.10–0.12	20–27	26.0 ± 1.7 SE	3.7 ± 0.58 SE	10.9 ± 0.51 SE	55.2 ± 1.96 SE	2.6 ± 0.46 SE
col-2	34	Colonial	0.08–0.12	19	27.8	9.3	16.3	44.1	0.4
col-2b$_2$ inosS1	23	Semicolonial	0.28–0.30	25–34	27.3	2.8	11.3	50.0	8.4
col-2b$_2$ inosS1	34	Colonial	0.13–0.16	34	26.6	4.1	16.4	47.8	1.8
col-2b$_2$ inosR11	23	Wild type	0.24–0.30	35	27.8	3.0	8.7	48.1	11.3
RL3-8A on 2% acetate	34	Semicolonial	0.16	34	22.4	3.1	5.4	61.5	7.3
Mixture of wild type and col-2				37	27.8	2.7	8.0	50.3	10.4

[a] All fatty acid values are given as mole per cent.

[b] Values are given in micromoles per gram of residual dry weight; they are from a previous publication (2), and are included here for ease of comparison.

[c] Results are expressed as total micromoles per gram of dry weight.

[d] Standard error.

TABLE 2. *Distribution[a] of fatty acids in phospholipids of various colonial mutants[b]*

Strain	Morphology	NADPH[c]	Fatty acids in phospholipids[d]	Palmitic	Stearic	Oleic	Linoleic	Linolenic
Balloon (sheared).....	Colonial	0.11–0.14	30	27.0	6.2	13.2	48.6	3.3
col-3 (sheared).....	Colonial	0.08–0.15	33	24.7	7.5	12.7	51.6	2.6
rg (sheared).....	Colonial	0.20–0.27	42	27.7	5.3	9.3	44.7	11.4
col-2 nt (sheared).....	Colonial	0.11–0.15	38	29.2	3.2	6.8	57.4	2.3

[a] All fatty acid values are given as mole per cent.
[b] All cultures were grown at 23 C.
[c] Values are given in micromoles per gram of residual dry weight; they are from a previous publication (2), and are included here for ease of comparison.
[d] Results are expressed as total micromoles per gram of dry weight.

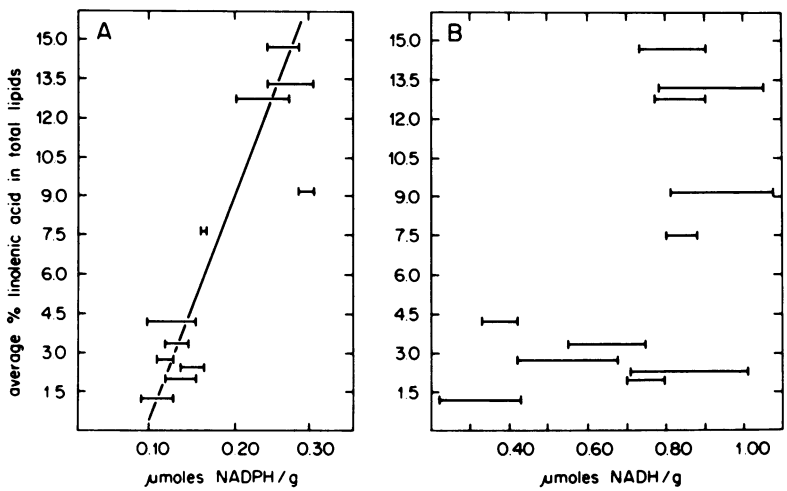

FIG. 1. *Relationship between mole percentage of linolenic acid and total content of reduced pyridine nucleotides. The values of NADPH and NADH were taken from a previous publication (2), and the NADPH values are listed in Tables 1 and 2 for easier comparison.*

the formation of linoleic acid, and that the presumed precursors, oleic and linolenic, accumulated somewhat owing to this block.

The levels of the three other fatty acids, stearic, palmitic, and palmitoleic, appeared to be similar in all strains, and the variations observed did not appear to correlate consistently with any morphological phenotype or with any given NADPH level. This is an encouraging result since it indicates that the col-2 mutation did not lead to changes in every fatty acid component of the phospholipid fraction, but possibly only interfered with the formation of linolenic acid. The level of palmitoleic acid was not included in the four tables since it showed little variation and comprised only 0.5 to 2.0 moles per cent of the total fatty acids.

Fatty acid variations in neutral lipids. The composition of the neutral lipid fractions is given in Tables 3 and 4. These extracts of col-2, col-3, and balloon also had less linolenic and more linoleic acid, and wild type grown on acetate (semicolonial growth) had an intermediary amount of linolenic acid. In general, the correlations based on the fatty acid composition of the neutral lipid fraction were consistent with the correlations found between the linolenic acid deficiency in the phospholipids and the NADPH levels. The large increase found in the total amount of neutral lipids in col-2 is puzzling and is currently under investigation.

Effect of certain parameters on fatty acid composition. The wild-type fatty acid composition of both the neutral lipids and phospholipids did not vary significantly with the age of the culture prior to the stationary, autolytic phase. Growth of the wild type as a liquid shake culture gave similar results to those previously obtained on standing

TABLE 3. *Distribution[a] of fatty acids in neutral lipids*

Strain	Growth temp	Morphology	Fatty acids in neutral lipids[b]	Palmitic	Stearic	Oleic	Linoleic	Linolenic
	C							
RL-3-8A	23	Wild type	33–66	22.8 ± 1.9 SE[c]	6.0 ± 0.95 SE	23.0 ± 0.50 SE	34.3 ± 0.87 SE	12.6 ± 1.35 SE
RL3-8A	34	Wild type		17.5	5.6	20.7	38.5	16.9
col-2	23	Colonial	116–200	29.7 ± 0.85 SE	8.6 ± 1.5 SE	16.1 ± 2.4 SE	40.5 ± 1.0 SE	3.1 ± 0.36 SE
col-2	34	Colonial	228	28.7	8.5	15.4	43.0	1.8
col-2b_2 inosS1	23	Semicolonial	27	18.1	12.4	16.0	42.3	10.1
col-2b_2 inosS1	34	Colonial	106	17.9	10.0	18.8	49.9	2.4
col-2b_2 inosR11	23	Wild type	54	19.9	5.2	21.1	37.3	15.2
RL3-8A, on acetate	34	Semicolonial	40	19.8	3.6	13.6	54.0	7.6
Mixture of wild type and col-2			179	29.3	5.1	18.3	41.3	4.9

[a] All fatty acid values are given as mole percent.
[b] Results are expressed as total micromoles per gram of dry weight.
[c] Standard error.

TABLE 4. *Distribution[a] of fatty acids in neutral lipids of various colonial mutants[b]*

Strain	Morphology	Fatty acids in neutral lipids[c]	Palmitic	Stearic	Oleic	Linoleic	Linolenic
Balloon (sheared)	Colonial	100	27.0	11.4	20.2	36.6	3.3
col-3 (sheared)	Colonial	92	27.0	8.7	18.3	39.9	5.4
rg (sheared)	Colonial	41	22.9	11.9	10.9	38.9	14.0
col-2 nt (sheared)	Colonial	268	31.1	3.5	15.1	45.9	1.7

[a] All fatty acid values are given as mole per cent.
[b] All cultures were grown at 23 C.
[c] Results are expressed as micromoles per gram of dry weight.

liquid cultures (6). No effect was noticed on either the wild-type or col-2 lipid composition when the lyophilization step was omitted. Storage of lyophilized mycelia at −15 C prior to methanol extraction did lower considerably the levels of linolenic acid found in any given culture. However, storage of the methanol extracts did not significantly affect any of the values obtained. Also, as seen in Table 1, the low levels of linolenic acid in col-2 were probably not due to certain types of extraction artifacts, since mixtures of approximately equal amounts of mycelia of col-2 and wild type gave intermediary amounts of linolenic acid upon extraction.

The observed differences in fatty acid composition between col-2 and wild type were found to be statistically significant. Table 5 indicates the number of individual determinations on both of these strains, and the probability that the differences between the respective mean values could have occurred by chance.

TABLE 5. *Statistical analysis of the differences in fatty acid distribution*

Lipids	No. of determinations		Calculated "t_s" values[a]	Probability[b] of chance occurrence
	Wild type	col-2		
				%
Phospholipids				
Linolenic	4	6	11.0	<1
Linoleic	4	6	3.0	<2
Oleic	4	6	4.5	<1
Neutral lipids				
Linolenic	4	6	8.9	<1
Linoleic	4	6	21.4	<1
Oleic	4	6	2.3	5

[a] Calculated as unpaired variates as given in *Handbook of Chemistry and Physics*, 48th ed., p. A160.
[b] Obtained from Tables in *Handbook of Chemistry and Physics*, 48th ed., p. A161.

DISCUSSION

The lower relative linolenic acid content observed in certain strains of *Neurospora* appears to be characteristic of only certain morphological mutants and not others. Mutants deficient in linolenic acid content are those that were previously shown to have a lower total NADPH content, as well as a lower NADH content (2). Therefore, it was not clear whether it was the NADPH deficiency or the NADH deficiency which lead to the observed fatty acid changes. As indicated in the Results section, there is an excellent correlation of linolenic acid level with NADPH content (Fig. 1A) and no obvious relationship with NADH content (Fig. 1B). Secondly, preliminary results (Brody, *unpublished data*) have indicated that the low levels of NADH found in many strains (balloon, col-2, col-3) can be increased to almost wild-type levels when nicotinic acid is added to the media, whereas the NADPH and linolenic acid contents remain low. On the basis of these correlations, one might predict that the in vivo conversion of linoleic to linolenic acid in *Neurospora* has a strict requirement for NADPH.

Since the levels of the other unsaturated fatty acids were not decreased by the NADPH deficiency, it might be argued that the other desaturation steps do not utilize NADPH in vivo, or else, can utilize both NADPH and NADH. Alternatively, the amount of NADPH needed for these steps might be far below the concentration required for the effective functioning of the final desaturation step, and, therefore, the 60% loss of NADPH might have little effect on these preceding steps. The fact that only linolenic acid was decreased might be explained if there was a separate NADPH-requiring enzyme which catalyzed the desaturation step, or if this reaction was carried out by an enzyme complex which utilized NADPH for the last step only. At the present time, the only enzymology that has been described for *Neurospora* is the report of a microsomal

preparation which catalyzed the conversion of stearic acid to linoleic acid and preferred NADH to NADPH for these first two desaturation steps (N. Baker and F. Lynen, *personal communication*).

The relationship between linolenic acid content and NADPH content, as expressed in Fig. 1A, could be interpreted to mean that the conversion of linoleic to linolenic acid was directly dependent on the NADPH level of the cell. An additional conclusion would be that, at normal steady-state conditions in the wild type, NADPH was probably below saturating concentrations in the vicinity of the linoleic acid desaturating enzyme, since a decrease in the level of NADPH led to a proportional decrease in linolenic levels. An extrapolation of the data in Fig. 1A indicates that there would be negligible linolenic production when the NADPH level falls to about one-third of the steady-state concentration of the wild type. One interpretation of these results would be that a critical concentration of NADPH is needed for any activity of this desaturating enzyme (i.e., a threshold effect). Another interpretation is that there are two separate pools of NADPH, one used for linolenic acid production and other reactions, and another pool which is not involved in this final desaturation step. This other NADPH might be compartmentalized elsewhere in the cell, or at least not readily accessible to the desaturating enzyme. In any event, the NADPH that is available for linolenic acid synthesis in vivo appears, for the most part, to be generated by the pentose phosphate shunt. A related and more extensive discussion of these points was given previously (2).

The contribution of the linolenic acid deficiency to the observed morphological changes is difficult to evaluate because attempts to reverse the morphology of col-2 with exogenous linolenic acid plus detergents were unsuccessful. It is clear that this deficiency is specifically related to those morphological mutants with low NADPH levels and could be considered a part of their syndrome. However, the linolenic deficiency need not be directly related to the morphological alterations since other pleiotropic effects produced by the NADPH decrease could lead to the abnormal shape and pattern formation that is observed. In any event, the ability to obtain membranes with altered composition might be a valuable tool in assessing the role of fatty acids in membrane permeability and stability.

Another interesting consideration for future studies would be an analysis of the amount of linolenic acid deficiency found among the various neutral and phospholipids of col-2, as well as its distribution between the α and β positions of the glycerides. In addition, a study of the effect of these mutations on the fatty acid composition of the various particulate constituents, mitochondria, etc., of this strain might prove worthwhile.

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