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Sterol Content and Enzyme Defects of Nystatin-Resistant Mutants of *Neurospora crassa*

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Summary. Non-saponifiable cell extracts of wild type and sterol mutants of *N. crassa* were analysed by means of gas-liquid chromatography. The wild-type contained ergosterol and episterol in a 10:1 ratio. None of the mutants was able to synthesize ergosterol. Three of the mutants carry single recessive gene mutations causing blocks in the terminal steps of ergosterol biosynthesis: *erg-1* has an inactive $\Delta^8 \rightarrow \Delta^7$ isomerase, *erg-2* has an inactive 24(28) hydrogenase, and *erg-4* has an inactive C-24 methyl transferase. Some of the mutants accumulated novel sterols as a result of their enzyme defects. The genes *erg-1* and *erg-2* were mapped close to *inl* on the right arm of chromosome V.

Introduction

Sterol mutants of *N. crassa* have been induced with ultraviolet radiation and chemical mutagens, and selected on media containing polyene antibiotics such as nystatin (Grindle, 1973, 1974). Most of the antibiotic-resistant mutants did not synthesize ergosterol, had a reduced rate of growth, and were female-sterile. In this paper, we are concerned primarily with the identification of the biochemical lesions in our sterol mutants, based on gas-liquid chromatographic analysis of non-saponifiable cell extracts. Two of the mutant genes have been mapped.

Materials and Methods

Details of the mutant phenotypes, and techniques used for their isolation, culture and genetic analysis have been described by Grindle (1973, 1974).

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For the gas-liquid chromatographic (GLC) studies, sterol extracts were prepared as follows: flasks of liquid media (Vogel, 1964) were inoculated with conidia and incubated at 26° C on a mechanical shaker; mycelia produced during the log phase of growth were harvested on Whatman No. 1 filter paper and washed with deionized water; non-saponifiable extracts of the mycelia were obtained as described by Woods (1971) except that heptane was replaced by spectrograde cyclohexane (Fisons Scientific, Loughborough, Leicestershire); the cyclohexane extracts were dried with a rotary evaporator, and the dried extracts were dissolved in 0.2 ml acetone prior to injection into the chromatographic columns.

All GLC studies were performed with a Pye series 104 chromatograph linked to a Bryans Southern 28,000 twin pen recorder. Extracts were analysed on 7 foot columns of (i) SE-30, 1% on Diatomite CLQ (100–120 mesh) at 240 and 249° C, (ii) OV–17, 3% on Diatomite CLQ (100–120 mesh) at 271° C, and (iii) OV–225, 3% on Diatomite CLQ (100–120 mesh) at 256 and 271° C (packaging materials from J.J. Chromatography Ltd., Kings Lynn, Norfolk). The carrier gas was argon, 60 ml per min., and relative retention times (RRT) were calculated relative to cholesterol (Sigma Chemical Co. Ltd., London).

The RRTs of Neurospora components were compared with those of authenticated components from yeast mutants (Woods and Bard, personal communication; see also Barton et al., 1974a, b, 1975) and with the gas chromatography data of Patterson (1971).

Results

Genetic Analysis. Two of the mutant genes, *erg-1* and *erg-2*, have been located on the right arm of chromosome V. Their linkage relationships (map units in brackets) are: centromere (?) *erg-2* (6) *inl* (6) *bis* (2) *erg-1* (9) *asp.*

Mutant types IV and VIII (see Grindle, 1974) carry the single recessive mutant genes *erg-3* and *erg-4*, respectively, affecting ergosterol biosynthesis. Neither of the genes is linked to the marker genes of the translocation strain "*alcoy*" and, consequently, they are not closely linked to *erg-1* or *erg-2*.

Only one isolate of mutant type V (see Grindle, 1973) was recovered. Although it was derived from conidia of the wild type 74-OR8-1 *a* by nitrous acid

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mutagenesis, it did not function as a mating type *a* parent in sexual crosses; surprisingly it generated numerous ascospores, of which about 70% were viable, when crossed as the male parent with 74-OR8-1*a*. The viable progeny varied considerably in rate of growth, morphology and sterol content, and few were phenotypically identical to the mutant parent i.e. compact growth, excellent conidiation, and type V sterol content. Offspring that functioned as *A* or *a* parents in sexual crosses occurred with equal frequencies, and segregation of the mating type alleles was independent of the genetic components affecting the other phenotypic characters. A small proportion of the progeny had the sterol phenotypes of the single-gene mutants *erg-1* and *erg-2* and exhibited the corresponding levels of resistance to nystatin. Thus, mutant V is a double mutant, *erg-1, erg-2*, and carries additional genetic lesions affecting mating type, rate of growth and morphology.

Mutant V behaved as a typical mating type *A* strain, and progeny from crosses involving this mutant functioned as either normal *A* or normal *a* isolates. Hence, the mating type gene seems to have mutated from *a* to *A*. There remains the possibility that mutant V was obtained inadvertently by mutating an existing sterol mutant; this seems unlikely, however, because it was isolated in one of the earliest mutation experiments, alongside many ergosterol mutants of mating type *a*, and before we had constructed sterol mutants of mating type *A* via genetic crosses.

Sterol Analysis. Representative isolates of the *erg-1, erg-2, erg-3* and *erg-4* mutants were selected, and each strain was cultured for GLC analysis on at least three separate occasions. Examples of the chromatograms from columns of OV-17 are given in Figure 1, and RRTs of sterol components on SE-30, OV-17 and OV-225 are given in Table 1. Our interpretation of the RRT data for the wild type and the *erg-1, erg-2* and *erg-4* mutants is given in Table 2.

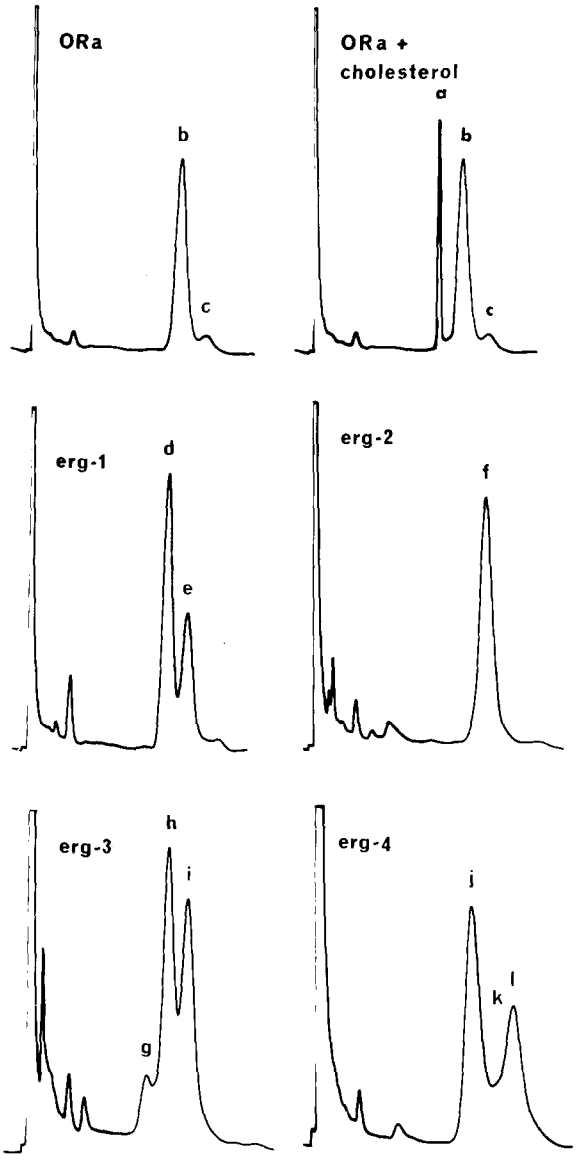

Fig. 1. Typical chromatograms from OV-17 columns of non-saponifiable cell extracts of wild type and sterol mutants of *Neurospora crassa*. Relative retention times of sterol components indicated are: **a**=1.00 (cholesterol), **b**=1.30 (ergosterol), **c**=1.52, **d**=1.19, **e**=1.39, **f**=1.49, **g**=1.04, **h**=1.22, **i**=1.40, **j**=1.33, **k**=1.49, **l**=1.66. Further resolution of peaks g and j is feasible (Table 1). ORa is the wild type 74-OR8-1*a* from which the mutants were derived

Discussion

The sterol phenotypes of the Neurospora strains indicate that three of the mutants are defective in enzymes or enzyme complexes involved in the terminal steps of ergosterol biosynthesis. They are compatible with a system proposed by Barton et al. (1973, 1974b) for ergosterol biosynthesis in yeast, and the probable gene/enzyme lesions are outlined in Figure 2. We interpret our observations on the ergosterol mutants as follows:

(i) *erg-4* strains are structural gene mutants with an inactive C-24 methyl transferase. The metabolic block prevents the introduction of the side chain methyl group, and the cells accumulate the immediate precursor zymosterol plus novel sterols (see Table 2) derived from zymosterol by the action of enzymes that normally interact with methylated components. This mutant is analogous to the *pol-1* mutant of yeast (Barton et al., 1974b).

(ii) *erg-1* strains are structural gene mutants with an inactive isomerase. The metabolic block prevents

Table 1. Relative retention times and relative peak heights of sterol components from wild type and ergosterol mutants of *Neurospora crassa*, separated on chromatographic columns of SE-30, OV-17 and OV-225

Column	Strain	Peak height				
		100%	95–75%	74–50%	49–20%	19–5%
SE-30	74-OR8-1*a*	1.220 ± 0.005				1.418 ± 0.006
	erg-1	1.137 ± 0.003	1.311 ± 0.003			
	erg-2	1.280 ± 0.002			1.396 ± 0.004	
	erg-3	1.428 ± 0.000			1.055 ± 0.000	
		1.244 ± 0.000				
	erg-4	1.199 ± 0.001				1.524 ± 0.002
OV-17	74-OR8-1*a*	1.297 ± 0.006				1.524 ± 0.008
	erg-1	1.193 ± 0.007		1.373 ± 0.009		
	erg-2	1.494 ± 0.007				
	erg-3	1.401 ± 0.001	1.222 ± 0.000		1.134 ± 0.001	
					1.039 ± 0.000	
	erg-4	1.655 ± 0.022	1.354 ± 0.003		1.494 ± 0.002	
			1.308 ± 0.002			
OV-225	74-OR8-1*a*	1.358 ± 0.006				1.604 ± 0.010
	erg-1	1.192 ± 0.003	1.414 ± 0.004			
	erg-2	1.640 ± 0.005				
	erg-3	1.453 ± 0.001	1.229 ± 0.001	1.355 ± 0.001	1.093 ± 0.000	
					1.007 ± 0.000	
	erg-4	1.521 ± 0.006		1.338 ± 0.006		1.778 ± 0.010

Retention times relative to cholesterol standard. Peak heights relative to the majr peak

Table 2. Sterol components in cell extracts of wild type and ergosterol mutants of *Neurospora crassa*

Strain	Major Sterols	Minor Sterols
74-OR8-1*a*	Δ_E5,7,22	Δ_E7,24(28)
erg-1	Δ_E5,8,22*	
	Δ_E8,24(28)	
erg-2	Δ_E5,7,22,24(28)	Δ_C8,24
		Δ_E7,24(28)
erg-4	Δ_C8,24	?Δ_C7,24?
	Δ_C5,7,24	
	Δ_C5,7,22,24	

* Principal component. The simplified nomenclature refers to cholestanes Δ_C and ergostanes Δ_E e.g. Δ_E5,7,22 is ergosta-5,7,22-trien-3β-ol (=ergosterol). Minor sterols are those with peak heights in gas chromatograms that are 75–95% lower than those of the most abundant sterol. Trace components have not been considered

$\Delta^8 \rightarrow \Delta^7$ isomerisation, and the cells accumulate the immediate precursor fecosterol plus the novel sterol, lichesterol, derived from fecosterol by the action of the dehydrogenase and hydrogenase enzymes that normally interact with Δ^7 substrates. The *erg-1* mutant is analogous to the *pol-2* mutant of yeast (Barton et al., 1974b) and is presumably allelic to the ergosterol mutants of *N. crassa* described by Morris et al. (1974).

(iii) *erg-2* strains are structural gene mutants with an inactive hydrogenase. The metabolic block prevents reduction of the 24,28 double bond, and cells accumulate the immediate precursor 24(28) dehydroergosterol plus two of the earlier precursors: episterol, that is a minor component of wild type cells; and zymosterol, that is a major component in *erg-4* mutants but does not occur in detectable amounts in the wild type. Wild strains of yeast can accumulate zymosterol (Barton et al., 1972) or 24(28) dehydroergosterol (Woods et al., 1974) but, in our normal strains of *N. crassa* and those analysed by Morris et al. (1974), zymosterol is evidentally metabolised rapidly and there is a corresponding build-up of episterol.

(iv) *erg-3* strains are not simple structural gene mutants that cause a block in the biosynthetic pathway outlined in Figure 2. There is no evidence for a defective 5,6 or 22,23 dehydrogenase, and our *erg-3* strains do not resemble the dehydrogenase mutants *pol-3* and *pol-5* described by Barton et al. (1974b). Some of the RRT data can be construed as indicating a defective $\Delta^8 \rightarrow \Delta^7$ isomerase, but our analytical method is inadequate for identifying those components with very low RRTs on the chromatographic columns (see Table 1) and, consequently, we have been unable to deduce whether *erg-3* strains are structural or regulatory gene mutants, or whether the biochemical lesion(s) affect enzyme(s) functioning before

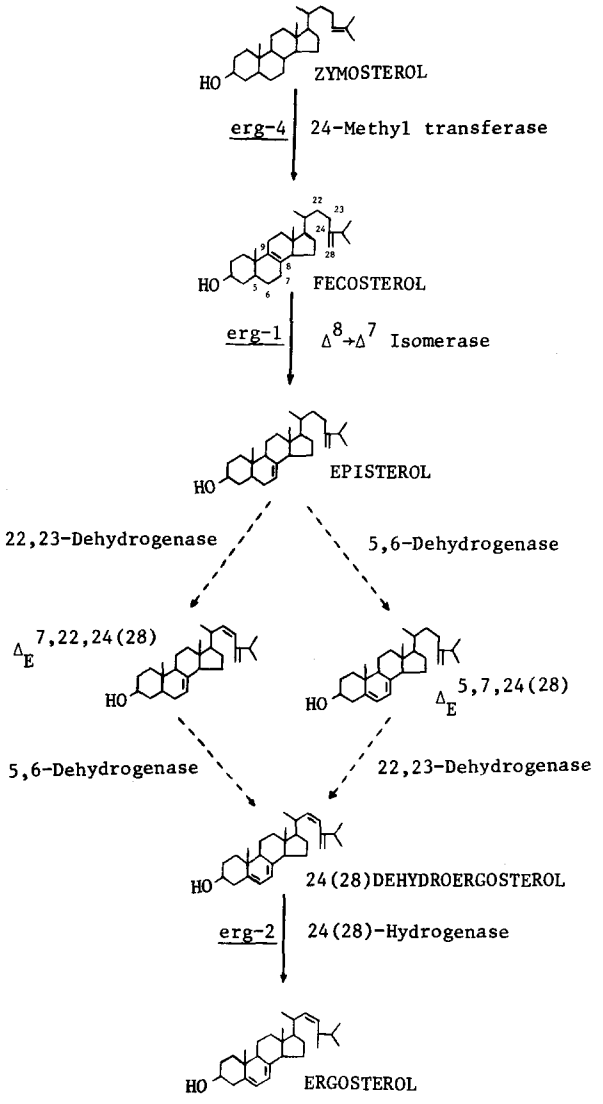

Fig. 2. A scheme for the terminal steps of ergosterol biosynthesis in wild type *Neurospora crassa*. The genes *erg-1*, *erg-2* and *erg-4* specify polypeptide components of the enzymes (or enzyme complexes) with the catalytic functions indicated

or after zymosterol synthesis. Whatever the *erg-3* gene's function, complementation studies indicate that the gene is not allelic to *erg-1*, *erg-2* or *erg-4*, and analysis of genetic crosses indicates that it is not closely linked to any of these *erg* genes. We hope to clarify the situation by constructing double mutants to assess interactions between *erg-3* and the other *erg* genes, and by processing cell extracts with more precise techniques such as mass spectrometry.

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