The Inducible Quinate-Shikimate Catabolic Pathway in *Neurospora crassa*: Genetic Organization*

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(Received 16 July 1973)

SUMMARY

In *Neurospora crassa* the conversion of quinate and shikimate to protocatechuate is catalysed by three inducible enzymes. Previous studies have demonstrated that the *qa-1* locus encodes a multimeric regulatory protein which is required for the synthesis of the three catabolic enzymes. The *qa-2* locus has been shown to encode catabolic dehydroquinase. This work defines two new classes of mutants. The *qa-3* locus is the structural gene for a bifunctional enzyme which catalyses the dehydrogenation of quinate and shikimate. Mutants of the *qa-4* class specifically lack 5-dehydroshikimate dehydrase. Alleles of the four different *qa* mutant classes are tightly clustered and are located to the left of *me-7* in L.G. VII.

INTRODUCTION

In contrast to the clustering found in prokaryotes, functionally related genes typically are dispersed throughout the eukaryotic genome (Demerec, 1964; Horowitz & Metzenberg, 1965). Few gene clusters have been defined in fungi and, with the apparent exception of the galactose system in yeast (Douglas & Hawthorne, 1964, 1966; Bassel & Mortimer, 1971), these clusters share two features which distinguish them from bacterial operons: the gene products are synthesized constitutively and these products remain physically associated as a multienzyme complex (Ahmed, Case & Giles, 1964; Fink, 1966; Giles, Case, Partridge & Ahmed, 1967*a*; Giles, Partridge, Ahmed & Case, 1967*b*; Berlyn, 1967; deLeeuw, 1967; Ahmed, 1968). Thus, the organization of the quinate-shikimate catabolic pathway in *Neurospora crassa* is unusual, since the enzymes of this pathway are highly inducible (Gross, 1958; Giles *et al.* 1967*b*; Doy, 1968) and apparently are not aggregated (Rines, 1969). This paper presents evidence which demonstrates that the four cistrons which control the inducible enzymes of the quinate-shikimate catabolic pathway are clustered in *N. crassa*.

In *Neurospora crassa* the conversion of quinate (QA) to protocatechuate (PCA) is catalysed by three enzymes which are induced in the presence of quinate and 5-dehydroquininate (Fig. 1) (Giles *et al.* 1967*b*; Rines, 1969). Quinate dehydrogenase (quininate:NAD oxidoreductase, EC. 1.1.1.24) catalyses the NAD-dependent oxidation of quinate to 5-dehydroquininate (DHQ). The conversion of DHQ to 5-dehydroshikimate (DHS) is mediated by a catabolic dehydroquinase (5-dehydroquininate hydro-lyase, EC. 4.2.1.10) (Giles *et al.* 1967*b*; Doy, 1968; Rines, 1969). DHS is subsequently dehydrated by dehydroshikimate dehydrase to form PCA (Tatum & Gross, 1956; Gross, 1958). Previous studies have suggested that a fourth activity, shikimate dehydrogenase (SDH), is coinducible and

* Adapted from a thesis submitted to the faculty of Yale University in partial fulfilment of requirements for the degree of Doctor of Philosophy.

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physically associated with quinate dehydrogenase (QDH) (Rines, 1969). The appearance of these four activities is dependent upon a multimeric regulatory protein which is encoded in the *qa-1* region (Valone, Case & Giles, 1971; Case, 1972; Partridge, Case & Giles, 1972). The *qa-1* locus is closely linked to *qa-2*, the structural gene for the catabolic dehydroquinase, in L.G. VII (Rines, 1969).

The quinate-shikimate catabolic pathway shares two intermediates in common with the aromatic biosynthetic pathway, DHQ and DHS (Fig. 1). In addition to the inducible, thermostable dehydroquinase, *Neurospora* possesses a biosynthetic dehydroquinase which is constitutive, thermolabile, and part of the multifunctional *arom* aggregate (Giles *et al.* 1967*b*; Rines, Case & Giles, 1969). The identification of these isozymes led Giles *et al.* (1967*b*) to propose that the *arom* aggregate functions as a channelling mechanism. The aggregate would segregate the metabolites common to both pathways and prevent catabolism of essential intermediates in the synthesis of the aromatic amino acids. The absence of catabolic dehydroquinase from strains possessing an intact aggregate and the induction of this enzyme in strains with a defective aggregate strongly suggests that the *arom* aggregate efficiently sequesters these common intermediates *in vivo*. Because of their many inter-relationships, any study of the quinate-shikimate catabolic pathway must also include consideration of the aromatic biosynthetic pathway.

METHODS

Strains. Most of the strains used in this study were supplied by Dr M. Case and were derived from, or are closely related to, wild-type 74A. Methionine auxotrophs, *me-7* (allele 4984) and *me-9* (allele NM43t), were obtained from the Fungal Genetics Stock Center (Humboldt State College, Arcata, California, U.S.A.). All *qa-1* and *qa-2* mutants were isolated by Rines (1969).

Media. Fries' minimal medium was used for all vegetative cultures. Fries' minimal medium containing 1.5 % sucrose will be referred to as 'sucrose minimal medium'. Similarly, Fries' minimal containing either quinate or shikimate as sole carbon source will be referred to as 'quininate minimal medium' and 'shikimate minimal medium', respectively. In media for plating, 0.05 % glucose + 0.05 % fructose + 1.0 % sorbose were substituted for sucrose to achieve colonial growth. This medium is referred to as 'sorbose medium'. Polyaromatic supplemented agar medium contained L-tyrosine 80 µg/ml, L-tryptophan 80 µg/ml, L-phenylalanine 80 µg/ml, and *p*-aminobenzoate 0.25 µg/ml. In liquid medium, a lower tryptophan concentration (16 µg/ml) was used. Other supplements and their concentrations in the media were: calcium pantothenate (2 µg/ml) and DL-methionine (100 µg/ml).

Mutant induction and selection. Seven-day-old conidia were irradiated with u.v. light according to the procedure of Giles (1951). Mutants unable to grow on quinate or shikimate were selected by filtration enrichment (Case, 1963). The isolation of phenotypic revertants of an *arom-1* strain was accomplished by plating irradiated conidia directly on to a sorbose medium supplemented with tyrosine and phenylalanine. Reversions of *qa-3* mutants were sought by scoring for growth on liquid 0.3 % quinate minimal medium. Tubes (12 × 100 mm) containing this medium were inoculated with 2000 to 4000 irradiated conidia. It is probable that each revertant represents a single mutational event since very few tubes contained growth.

Complementation tests. These tests were made by inoculating liquid minimal medium with a conidial suspension of each pair of mutants.

Growth of mycelia for extraction. Fresh conidia (5 to 7 days old) were suspended in

water and filtered through cotton. This conidial suspension was used to inoculate a 2 l Erlenmeyer flask containing 500 ml of medium (to a final concentration of 10^6 conidia/ml medium). The culture was incubated at 25 °C for 30 h on a gyratory shaker (New Brunswick Scientific Co.) at 300 rev./min.

'Shift cultures' were harvested on a sterilized Buchner funnel and washed with 500 ml sterile minimal medium (no carbon source). The mycelia were resuspended in 500 ml of fresh medium containing the indicated carbon sources. The transferred cultures were grown on shakers at 25 °C for the designated time periods.

Preparation of cell-free extracts. Initially, mycelia were extracted according to the procedure of Giles *et al.* (1967*a*), with the exception that tris-HCl buffer was used in lieu of K_2PO_4 buffer and desalting was accomplished with a Sephadex G-25 column (Procedure I). However, another procedure (II) was found to be more useful for obtaining quantitative data. Harvested mycelia were lyophilized and extracted in 50 mM-tris-HCl buffer, pH 7.1, containing 1 mM-EDTA and 1 mM- α -thioglycerol. Cellular debris was removed by centrifugation at 27000 *g* for 30 min. The supernatant was withdrawn from beneath the surface lipid layer and centrifuged at 105000 *g* for 2 h at 4 °C in a Beckman L2-65 ultracentrifuge. The lipid layer was pipetted and discarded and the clear supernatant assayed directly.

Enzyme assays. All assays were performed spectrophotometrically at 37 °C in a total volume of 0.60 ml in silica cuvettes of 1.0 cm light path. Specific activities are defined as nmol of substrate consumed (or of product formed)/min/mg protein. Protein concentrations were determined by the biuret method of Mokrasch & McGilvery (1956). Published methods were used for the assay of dehydroshikimate reductase (Giles *et al.* 1967*a*) and protocatechuate oxygenase (Case, Giles & Doy, 1972). Quinate dehydrogenase activity was measured by following the reduction of NAD in a reaction mixture containing 85 mM-tris-HCl (pH 8.6), 6.7 mM-quinatate, 2.5 mM-NAD, 0.3 mM-KCN, and 1/30 vol. extract. Shikimate dehydrogenase was measured by the same procedure as quinate dehydrogenase except that 15.7 mM-shikimate was used as substrate. Catabolic dehydroquinase activity was assayed in a reaction mixture containing 90 mM-tris-HCl (pH 7.4), 0.2 mM-EDTA, 0.67 mM-DHQ and 1/30 vol. enzyme preparation; before assaying, the enzyme extract was heated at 70 °C for 10 min and centrifuged to remove the biosynthetic dehydroquinase and DHS dehydrase. Dehydroshikimate dehydrase was measured in a reaction mixture containing 90 mM-tris-HCl (pH 7.4), 0.2 mM-EDTA, 0.67 mM-DHQ, and 1/30 vol. heat-treated (10 min at 70 °C) enzyme preparation containing catabolic dehydroquinase activity. The substrate (DHS) was generated *in vitro* during 45 min incubation. The formation of protocatechuate was followed in the presence of argon at 290 nm.

Under these conditions of assay, QDH, SDH, catabolic dehydroquinase and DHS dehydrase activities display a linear dependence on protein concentration (Chaleff, 1972).

Thermolability experiments. Equal volumes of an enzyme preparation were incubated at the appropriate temperature ± 0.5 °C, samples removed at time intervals and centrifuged at 17500 *g* for 20 min before assay.

Chemicals. Purified crystalline DHQ was generously supplied by Dr C. W. H. Partridge. D-Quinate was purchased from K & K Laboratories, Plainview, New York, U.S.A. Shikimate and protocatechuate were products of Calbiochem.

Table 1. *Enzyme activities in qa mutant strains*

Genotype	Strain	Specific activities in enzyme extracts*					
		QDH	SDH	Catabolic DHQase	DHS dehydrase	PCA oxygenase	DHS reductase
Wild type	74A	56.7	163.1	56.7	14.5	24.3	—
<i>qa-3</i>	M2-1A	0	4.2	168.2	93.1	5.5	—
<i>qa-3</i>	M16-1A	0	6.1	197.0	98.9	3.6	54.3
<i>qa-3</i>	M17-1A	0	4.4	180.2	85.8	6.1	—
<i>qa-3</i>	M22-1A	0	6.5	163.3	92.9	1.6	30.1
<i>qa-3</i>	M26-1A	0	5.8	154.5	90.7	2.0	38.3
<i>qa-3</i>	M29-1A	0	8.0	148.1	96.1	1.4	40.1
<i>qa-3</i>	M33-1A	0	7.8	173.7	103.4	2.5	37.3
<i>qa-3</i>	M45-1A	0	5.7	174.7	100.6	2.2	40.9
<i>qa-3</i>	M59-1A	0	10.2	198.1	77.2	1.8	—
<i>qa-3 arom-1</i>	M2-1183A	0	0	666.1	114.9	4.9	0
<i>qa-3 arom-1</i>	M16-1183A	0	0	688.8	110.3	6.5	0
<i>qa-3 arom-1</i>	M22-1183A	0	1.2	690.1	123.4	3.8	0
<i>qa-3 arom-1</i>	M33-1183A	0	0	672.1	111.7	2.0	0
<i>qa-4</i>	M18-1A	227.0	681.9	180.3	0	0.3	—
<i>qa-4 pan-2</i>	RR3-B36-1A	123.3	348.7	64.8	5.7	4.4	24.0
<i>qa-4 pan-2</i> <i>arom-1</i>	RR57-B36-1A	117.4	298.4	93.7	0	0	6.0
<i>qa-4 pan-2</i>	RR70-B23-1A	123.1	334.0	65.0	0.5	0	23.7
<i>qa-4 pan-2</i>	RR134-B36-1A	108.0	314.5	56.0	0.9	0.6	33.7
<i>qa-4 pan-2</i>	RR135-B23-1A	273.9	836.2	221.0	0	0	22.6
<i>qa-4 pan-2</i>	RR164-B36-1A	182.7	423.8	142.0	0	0.5	27.1
<i>qa-1^s arom-9</i>	I40-M6-11A	0.7	5.6	0.7	0.5	0.5	—
<i>qa-2 pan-2</i>	203-B23A	54.4	164.9	0	16.8	20.7	—
<i>qa-2 arom-9</i>	203-M6-11A	266.4	698.8	0	41.1	0.8	—
<i>qa-2 arom-9</i>	246-M6-11A	241.6	644.6	0	31.1	0.6	—
<i>qa-2 qa-4</i> <i>arom-1</i>	RR228A†	166.6	564.9	0.2	2.8	2.5	—
<i>qa-3 me-7</i> <i>arom-9</i>	M16-4894- M6-11A	0.4	7.7	205.7	116.1	1.9	—
<i>qa-4 me-7</i> <i>arom-9</i>	M18-4894- M6-11A	143.8	422.9	107.5	0	0.4	—

* Mycelia were grown on appropriately supplemented 1.5 % sucrose medium for 30 h and were shifted to identically supplemented 0.3 % quinate medium for 5 h. Extraction was by Procedure II.

† This strain was not outcrossed before extraction.

RESULTS

Recovery and identification of mutants

An initial selection of 200 isolates resulted from a filtration enrichment experiment (Y330) in which Dr M. Case selected against growth of wild-type conidia from strain 74A on 0.3 % quinate. Those isolates which were able to grow on *p*-hydroxybenzoate as sole carbon source, but not on quinate, were tested for their complementation responses with known *qa-1* and *qa-2* strains. Enzyme assays of complementing isolates revealed one mutant which specifically lacked DHS dehydrase (M18) and nine which were deficient for both QDH and SDH activities (Table 1). Mutants which are deficient for both QDH and SDH activities were designated *qa-3*. The isolate which lacks DHS dehydrase activity defined the *qa-4* locus. Other extensive filtration enrichment experiments with shikimate as sole carbon source utilizing wild-type (74A) and *arom-1* (1183) strains failed to yield additional *qa* mutants.

Case *et al.* (1972) found that the introduction of the *qa-4* mutant into an *arom-1* strain (which lacks DHS reductase activity) results in the physiological suppression of the *arom-1*

Table 2. *A comparison of catabolic enzyme activities in several strains induced on quinate and shikimate*

Genotype	Allele	Inducer	Specific activities in enzyme extracts*			
			QDH	SDH	Catabolic DHQase	DHS dehydrase
Wild type	74A	None	0.4	12	0	0.2
Wild type	74A	0.3 % QA	204	560	223	100
Wild type	74A	0.3 % SA	41	159	62	22
<i>qa-3</i>	M16-1A	0.2 % SA	0	20	69	23
<i>qa-3</i>	M22-1A	0.2 % SA	0	16	95	32
<i>qa-1^F pan-2</i>	159; B23a	0.3 % QA	37	110	115	32
<i>qa-3⁺ arom-1</i>	M16 ⁺ ; 1183a					

* Mycelia were grown on 1.5 % sucrose minimal medium for 30 h and were shifted to medium containing the designated inducer for 8 h. Extraction was by Procedure I.

phenotype. This suggested that *qa-4* mutants could be recovered as apparent revertants of an *arom-1* strain. In one 'reversion' experiment 300 isolates were obtained which would grow on sucrose minimal medium but not on a polyaromatic quinate medium. Initially it was assumed that all these strains were *qa-4 arom-1* double mutants.

Case *et al.* (1972) demonstrated that an unusually high reversion frequency could be achieved in *arom-1* strains and in *arom-1 qa-2* double mutants, but not in either *arom-1 qa-1* or *arom-1 qa-3* double mutants. This evidence suggested that the products of the *qa-3* and *qa-1* genes, but not that of the *qa-2* gene, are required to produce the suppressed phenotype. Therefore, 250 *arom-1* revertants were tested further by complementation with a *qa-2* strain to determine whether any of these isolates also lacked the catabolic DHQase. Only one isolate, RR228A, did not complement the *qa-2* strain. Another isolate, RR231A, showed reduced complementation with the *qa-2* tester and little complementation with the *qa-3* strain.

Revertants of *qa-3* strains were sought in an attempt to define the structural gene(s) encoding the QDH and SDH activities. Several isolates were recovered which had regained the ability to grow on quinate. Two of these isolates, M16-1A-RV3 and M22-1A-RV1, were temperature-sensitive: both were able to grow on sucrose minimal medium at 25 and 35 °C, but grew on quinate minimal medium only at 25 °C.

Complementation relationships among qa mutants

Rines (1969) distinguished two classes of *qa-1* mutants on the basis of their different complementation responses with *qa-2* mutants. Alleles of the class designated *qa-1^F* complement *qa-2* mutants rapidly, whereas complementation between *qa-2* and *qa-1^S* mutants is much slower and in some instances barely detectable after 20 to 30 days. In the present studies, 17 *qa-2* and 25 *qa-4* alleles and all nine *qa-3* alleles complemented rapidly with *qa-1^F* strains and only very slowly with a *qa-1^S* isolate. Thus, members of the two *qa-1* mutant classes exhibit the same functionally distinct responses in complementation tests with members of all three of the other *qa* mutant classes.

In addition to complementing mutants of the *qa-1^F* class, mutants of any one of the three singly deficient classes, *qa-2*, *qa-3* and *qa-4*, readily complement members of the other two mutant classes on quinate medium. A different complementation pattern emerged from tests on shikimate medium. Whereas *qa-3* mutants complement rapidly with *qa-1^F* and slowly with *qa-1^S* strains on quinate, these strains do not complement on shikimate. A *qa-4* isolate

Fig. 1. Diagram of the reactions in the polyaromatic synthetic (constitutive) and the quinate-shikimate catabolic (inducible) pathways in *Neurospora crassa*.

complemented both the *qa-1^F* and the *qa-3* strains equally well on both carbon sources. The failure of the *qa-1* and *qa-3* isolates to complement on shikimate suggested either that these two mutant classes may not define genetically distinct loci or that mutational events in one region may influence the expression of the other region. Enzyme assays, however, showed that the inducibility of *qa-3* mutants on shikimate (SA) was not impaired, since the catabolic enzymes were present in extracts of two *qa-3* strains which were induced on 0.2 % shikimate minimal medium for 8 h (Table 2). The presence of SDH activity in a quinate-induced heterokaryon forced between a *qa-1^F* and a *qa-3* strain demonstrates that the *qa-3* region *cis* to the *qa-1* mutation produces an active enzyme (Table 2).

No complementation was observed among isolates from backcrosses of the nine *qa-3* alleles when tested in all possible pairwise combinations on either quinate or shikimate medium.

Enzyme assays

The nature of the genetic deficiencies in the mutant strains was determined directly by assaying the levels of QDH, SDH, catabolic DHQase, DHS dehydrase, and PCA oxygenase in extracts. PCA oxygenase is induced only in the presence of PCA and several analogues not normally found in the cell (Gross & Tatum, 1955; Gross, Gafford & Tatum, 1956). Since extracts of *qa-1 arom-9* and *qa-2 arom-9* mutants which have been exposed to quinate are

Table 3. Enzyme activities in several *qa-4* isolates

Genotype	Strain	Specific activities in extracts*			
		QDH	Catabolic DHQase	PCA oxygenase	Ratio DHQase:QDH
<i>qa-4 arom-1</i>	RR13A	139.1	154.3	0.5	1.11
<i>qa-4 arom-1</i>	RR19A	124.2	157.1	8.8	1.26
<i>qa-4 arom-1</i>	RR21A	138.9	148.8	1.0	1.07
<i>qa-4 arom-1</i>	RR53A	131.0	77.1	0	0.59
<i>qa-4 arom-1</i>	RR159A	142.0	160.3	0	1.12
<i>qa-4 arom-1</i>	RR228A	148.7	0.2	1.5	—
<i>qa-4 arom-1</i>	RR231A	6.6	50.5	7.9	7.65
<i>qa-4 pan-2</i>	RR70-B23-1A†	79.4	54.3	0	0.68
<i>qa-4 pan-2</i>	RR134-B36-1A†	82.6	55.8	0	0.68
<i>qa-4</i>	MI8-1A†	176.4	142.8	0.6	0.81
Wild type	74A‡	56.7	56.7	24.3	1.00

* Mycelia were grown on 1.5 % sucrose polyaromatic medium for 30 h and were shifted to 0.3 % quinate polyaromatic medium for 5 h. All strains containing a *pan* marker were grown on medium additionally supplemented with calcium pantothenate. Extraction was by Procedure II.

† These strains were outcrossed before extraction.

‡ Data from Table 2.

deficient for PCA oxygenase as well as for at least one activity prior to PCA in the catabolic pathway (Table 1), it is clear that the formation of PCA from quinate and DHQ can be accomplished only by the reaction sequence shown in Fig. 1. Thus, the levels of PCA oxygenase in extracts may be used as a quantitative measure of the rate of flow of intermediates through the catabolic pathway *in vivo*.

Strains derived from backcrosses of the original nine *qa-3* isolates to wild type lacked QDH activity and possessed low, but measurable, SDH activity (Table 1). Most, if not all, of the SDH activity was absent from strains which contained these same *qa-3* alleles in an *arom-1* background (Table 1). It is probable, therefore, that the residual SDH activity in the *qa-3 arom-1*⁺ strains resulted from interference in the SDH assay by DHS reductase.

DHS dehydrase activity was reduced or undetectable in extracts of *qa-4* mutants (Table 1). The corresponding decrease or absence of PCA oxygenase activity in these strains would suggest that the conversion of DHS to PCA could be accomplished only by DHS dehydrase. Nine mutants which were isolated as phenotypic revertants of an *arom-1* strain and which displayed a reduced complementation response with a *qa-2* tester were assayed for DHQase, QDH and PCA oxygenase (Table 3). The absence or reduced level of PCA oxygenase activity indicated that these mutants were unable to convert DHS to PCA. Mutant RR228A had no catabolic DHQase activity and barely detectable levels of PCA oxygenase. Subsequent assay confirmed that this strain was deficient for both catabolic DHQase and DHS dehydrase (Table 1). Mutant RR231A had only trace amounts of QDH and low levels of DHQase. The low PCA oxygenase level suggests that some DHS dehydrase activity is present *in vivo*. Table 3 also lists the ratio of catabolic DHQase to QDH in these *qa-4 arom-1* isolates. In extracts of a number of strains grown under a wide variety of conditions this ratio is consistently 1.0 ± 0.2 (Chaleff, 1974). Deviations from this value would be expected in mutants in which catabolic DHQase synthesis is reduced by a polar nonsense mutation in the *qa-4* gene. Isolates RR53A, RR70-B23-1A and RR134-B36-1A had low DHQase/QDH ratios (also see Table 1). Whether these ratios were caused by genetic pleiotropy or whether they resulted from secondary physiological effects can be determined only by additional analyses.

Table 4. *Analysis of random isolates from crosses between qa mutants and me-7*

Cross	No. of isolates	No. of pseudo wilds	No. of recombinants		Recombination* (%)
			<i>qa</i> ⁺ <i>me-7</i> ⁺	<i>qa me-7</i>	
<i>qa-3</i> (M16)A × <i>me-7a</i>	2114	24	0	2	0.09
<i>qa-4</i> (M18)A × <i>me-7a</i>	2047	20	0	1	0.05

* Percentage recombination is defined as (total no. of recombinants/total no. isolates) × 100.

Table 1 presents a comparison of the enzyme activities in representative strains of the four *qa* mutant classes. It should be noted that *qa-3* and *qa-4* mutants had much higher levels of the intact catabolic enzymes than did the wild type. Two *qa-2 arom-9* strains also showed enzyme levels higher than wild-type, but when one of these *qa-2* alleles was crossed into a *pan-2* background, the enzyme levels became equal to those of the wild-type strain. PCA oxygenase activity, which was barely detectable in the *qa-3*, *qa-4* and *qa-2 arom-9* strains, was also present in the *qa-2 pan-2* isolate at wild-type levels. The higher enzyme levels in the mutants were probably caused by the higher endogenous concentrations of inducer which resulted from the inability of these strains to catabolize quinate. The presence of wild-type levels of PCA oxygenase in the *qa-2 pan-2* strain indicates that it is able to catabolize quinate, presumably via the biosynthetic dehydroquinase, and as a result, the enzyme levels are similar to those of wild type. These results are consistent with the observation that the *qa-2 pan-2* strains display slight growth on pantothenate-supplemented quinate medium whereas the *qa-2 arom-9* strains do not grow at all on polyaromatic quinate medium.

Genetic mapping. Random spore analysis

Conditions of plating which permit wild-type and mutant spores to be distinguished by a growth rate differential have not been defined (Chaleff, 1972). For this reason preliminary mapping was achieved by isolating and analysing random spores from a population. All prototrophs from those crosses which involved the closely linked *me-7* or *me-9* markers were screened to eliminate pseudo wild types by plating conidia on non-selective (methionine-supplemented) and selective (minimal) media. Viability of all crosses from which random spores were selected was approximately 100 %.

Crosses with me-7

The results of an analysis of random spores derived from the crosses *qa-3*(M16)A × *me-7a* and *qa-4*(M18)A × *me-7a* are presented in Table 4. Double mutants of the type *qa-3*(M16) *me-7* and *qa-4*(M18) *me-7*, isolated from these crosses, were used in subsequent mapping experiments. The genotypes of the double mutants were confirmed by complementation tests and by enzyme assays (Table 1). These results demonstrate the close linkage of *qa-3* and *qa-4* to *me-7* in L.G. VII.

Three-point crosses

Additional genetic markers were introduced into the two double-mutant strains *qa-3*(M16) *me-7* and *qa-4*(M18) *me-7*. The derived strains, *qa-3 me-7 cot a* and *qa-4 me-7 cot al₂* were crossed to *qa-r^s* (140) *cot al₂ pan-2A* and random isolates were recovered from a plating of spores on sorbose medium supplemented with pantothenate and methionine. The isolates were screened for *qa*⁺ prototrophs on supplemented liquid quinate medium. The results in Table 5 suggest that the *qa-3* and *qa-4* genes are located 0.016 units (where units designate percentage prototrophs) and 0.080 units, respectively, to the right of *qa-r^s*. This interpreta-

Table 5. Random isolates from the three point crosses *qa-1^s × qa me-7*

Cross	No. of isolates	<i>qa⁺</i> prototrophs		Prototroph frequency (%)
		<i>me-7⁺</i>	<i>me-7</i>	
<i>qa-1^s(140) cot al₂ pan-2A × qa-3(M16) me-7 cot a</i>	6287	1	0	0.016
<i>qa-1^s(140) cot al₂ pan-2A × qa-4(M18) me-7 cot al₂ a</i>	5031	3	1	0.080

Table 6. Three-point crosses: *qa me-7A × me-9a*

Cross	Number of ascospores		Germination (%)	No. of pseudo wilds	<i>me⁺</i> prototrophs		Prototroph frequency* (%)
	Total	Viable			<i>qa⁺</i>	<i>qa</i>	
<i>qa-3(M16) me-7A × me-9a</i>	376 282	263 581	70	27	8	2	0.004
<i>qa-4(M18) me-7A × me-9a</i>	231 990	179 227	77	13	18	8	0.015

* The calculation of the prototroph frequency is based on the number of viable spores plated.

tion assumes that the one *qa⁺ me-7* isolated from the cross *qa-4 me-7 × qa-1^s* resulted from a conversion event. An interpretation in which the origin of this isolate is attributed to reciprocal recombination would lead to a genetic map in which *me-7* is situated between *qa-3* and *qa-1^s*.

Selection for prototrophs on sorbose minimal medium

Crosses with *me-9*. Murray (1970) has shown that *me-9* is adjacent to, and to the right of, *me-7* in L.G. VII. To define the position of the *qa* genes with respect to the *me-7* and *me-9* loci, the *qa me-7* double mutants were crossed with a temperature-sensitive *me-9* allele (NM43t). Segregation of the *qa* allele was followed in recombinants between the *me-7* and *me-9* markers which were selected on sorbose minimal medium at 35 °C (Table 6). The recovery of true prototrophs from these crosses was hindered by a large frequency of pseudo wild formation (all isolates were screened by conidial platings). The fourfold difference between the prototroph frequencies from the two crosses may be due to differences in genetic constitution between the two double-mutant strains. Evidence has suggested that the extent of genetic heterogeneity affects the frequency of gene conversion (Fogel, Hurst & Mortimer, 1971). Determination of gene order from segregation of the unselected *qa* marker is complicated by the fact that approximately 75 % of the methionine prototrophs result from gene conversion; furthermore, these conversion frequencies exhibit a marked polarity pattern (Murray, 1970). In view of this consideration, the results in Table 6 are consistent with a genetic map in which both *qa-3* and *qa-4* are located to the left of *me-7*.

Crosses with *qa-1^s (140) arom-9A*. The absence of a functional dehydroquinase from both *qa-1 arom-9* and *qa-2 arom-9* strains allowed Rines (1969) to construct a preliminary genetic map from the number of prototrophs which appeared among spores plated on sorbose minimal medium. Because the same *arom-9* allele was used in all strains, elimination of the polyaromatic requirement could be achieved only by recombination between the *qa* alleles. This technique was used to determine the genetic distance between a *qa-1^s* and a *qa-2* allele (Table 7).

The frequency of recombination between *qa-1* and *me-7* may be determined by plating spores from the cross *qa-1 arom-9 × me-7 arom-9* on sorbose minimal medium (Rines, 1969).

Table 7. Prototroph frequency in crosses of *qa-1^s × qa me-7* and *qa-1^s × qa-2*

Cross	No. of ascospores		Germination (%)	No. of prototrophs		Prototroph frequency*	
	Total	Viable		<i>qa⁺</i>	<i>qa</i>	% of total no. of spores	% of no. of viable spores
<i>qa-1^s(140) arom-9A</i> × <i>qa-3(M16) me-7 arom-9a</i>	97 332	49 752	51·1	13 (22 %)	46 (78 %)	0·061	0·119
<i>qa-1^s(140) arom-9A</i> × <i>qa-4(M18) me-7 arom-9a</i>	244 650	91 040	37·2	29 (34 %)	57 (66 %)	0·035	0·094
<i>qa-1^s(140) arom-9A</i> × <i>qa-2(246) arom-9a</i>	182 120	84 950	46·6	150	—	0·082	0·177

* Prototroph frequencies calculated on the basis of the estimated total number of spores plated apparently are more meaningful than are frequencies calculated from the number of viable spores plated (Rines, 1969). This probably results from a differentially greater viability of the recombinant spores.

Incorporation of the *arom-9* allele into the double mutants *qa-3 me-7* and *qa-4 me-7* makes possible three-point mapping in which *qa-3* and *qa-4* are the unselected markers. Almost all prototrophs obtained from crosses of these strains with the slow-complementing *qa-1^s(140) arom-9A* strain were true wild types (Table 7), but all prototrophs from crosses of strains *qa-3 me-7 arom-9a* or *qa-4 me-7 arom-9a* with either *qa-2 arom-9A* or *qa-1^F(159) arom-9A* were pseudo wilds. The preponderance of pseudo wilds prohibited an analysis of these crosses.

Different prototroph frequencies are obtained from crosses of the *qa-3 me-7 arom-9a* and *qa-4 me-7 arom-9a* strains with the *qa-1^s arom-9A* strain although both, presumably, represent the frequency of recombination events in the same region *qa-1^s me-7*. It is therefore apparent that a direct comparison between the two crosses of frequencies of the *qa⁺* prototrophs would not be useful for determining the distance between the *qa-1^s* and the *qa-3* and *qa-4* markers. The frequency with which each class of prototrophs (*qa⁺* and *qa*) is recovered within the same cross should, however, reflect the relative lengths of the two regions *qa-1^sqa* and *qame-7*. In effect, a comparison of the frequencies of the two prototroph classes obtained from a given cross normalizes the frequencies and permits a comparison to be made between crosses. The percentages of *qa* and *qa⁺* prototrophs obtained in each cross (Table 7) are consistent with the order: *qa-1^s qa-3 qa-4 me-7*.

A preliminary genetic map of the *qa* region has been constructed from these results (Fig. 2). The relative positions of *qa-2* and *qa-4* are still uncertain because although the *qa-1 qa-2* distance was determined from a large number of prototrophs the *qa-1 qa-4* distance is based on only four prototrophs.

Linkage of the temperature-sensitive qa-3 revertant, M16-1A-RV3, to me-7

To determine whether the temperature-sensitive revertant phenotype of strain M16-1A-RV3 was due to a mutation of the *qa-3* gene, the revertant was tested for linkage to *me-7*. Random *me-7⁺* spores from the cross M16-1A-RV3 × *me-7a* were scored for growth on liquid quinate medium at 25 and 35 °C. No prototrophs were recovered among 212 spores isolated. These results indicate that the temperature-sensitive revertant is linked closely to *me-7* and may well be within the *qa-3* locus.

Fig. 2. Preliminary genetic map of the *qa* cluster. Numbers represent the percentage of prototrophs arising in crosses between the alleles. The distances reported for the *qa-1^s qa-3* and *qa-1^s qa-4* region are based on random spore analyses. The *qa-1^s qa-2* distance was determined by direct selection for prototrophs among a spore population.

Thermolability of QDH and SDH activities

In earlier studies, Rines (1969) detected an NAD-specific shikimate dehydrogenase activity which is coinducible with quinate dehydrogenase. The SDH activity is also absent from *qa-1* mutants. Inability to separate QDH and SDH activities led Rines (1969) to suggest that they are associated with a single protein. The simultaneous loss of both dehydrogenase activities as the result of single mutational events (nine *qa-3* mutants) provided the first genetic evidence supporting the hypothesis that the *qa-3⁺* gene product is a bifunctional protein. It appeared possible, however, that these genetic lesions occurred in either a regulatory element or in a locus encoding a common polypeptide essential for the expression of both activities. Therefore the rates of thermal denaturation of QDH and SDH activities in wild-type strains and in revertants of *qa-3* mutants were examined.

The kinetics of thermal inactivations at 45 °C of QDH and SDH were nearly identical in extracts of wild-type (74A) cells which had been induced on either quinate (Fig. 3*a*) or shikimate (Fig. 3*b*). In the quinate-induced extract the half-life of both activities was approximately 5.0 min. The approximate half-life was 5.4 min in the shikimate-induced extract. Although activities disappeared at very nearly the same rate in both extracts, the SDH activity appeared to be slightly more stable. The presence of residual SDH activity in *qa-3* strains and its absence from *qa-3 arom-1* strains (Table 2) suggested that DHS reductase interferes in the SDH assay. To determine whether the apparently greater stability of SDH at 45 °C in wild-type extracts is due to interference by the biosynthetic enzyme, the thermolability experiments were repeated with an *arom-1* strain (1183) which is mutant for this activity. Fig. 4 indicates that at 43 and 45 °C, QDH and SDH displayed identical denaturation kinetics. These results are consistent with the hypothesis that a common enzyme catalyses the oxidation of quinate and shikimate in *Neurospora*.

To determine whether *qa-3⁺* is the structural gene for QDH and SDH, the thermolability properties of quinate and shikimate dehydrogenase activities recovered in revertants of *qa-3* mutants were examined. Neither QDH nor SDH activity was detectable in extracts of several revertants. In all revertants which possess measurable activity, both dehydrogenase activities have been regained. The QDH and SDH activities present in extracts of three revertants were indistinguishable from the wild-type activities by thermolability criteria (Chaleff, 1972). SDH and QDH activities in a fourth revertant, M16-1A-RV3, disappeared coincidentally at a rate which was different from that at which the wild-type activities were lost (Fig. 5). At 37 °C the approximate half-life of the mutant enzyme was 18 min, and that for the wild type was 53 min. The close agreement between the expected and the actual denaturation kinetics of a mixture of equal parts of wild-type and revertant extracts demonstrates that the proteins are behaving independently and that the increased lability of the revertant

Fig. 3(a). For legend see facing page.

protein is not caused by a soluble molecule. Additional evidence which suggests that a modified protein catalyses the oxidation of quinate and shikimate in revertant MI6-1A-RV3 is provided by the altered SDH/QDH ratio. In wild type, the ratio of shikimate dehydrogenase activity to quinate dehydrogenase activity is 2.5 to 3.0 (Chaleff, 1974). In two separate experiments this ratio was shown to be 1.9 to 2.0 in enzyme preparations of MI6-1A-RV3. It is apparent from these studies that a single enzyme, which is encoded in the *qa-3⁺* gene, catalyses the dehydrogenation of both quinate and shikimate.

DISCUSSION

The tight clustering of four cistrons involved in the catabolism of quinate and shikimate in *Neurospora crassa*, *qa-1*, *qa-2*, *qa-3* and *qa-4*, has been established. The *qa-2* and *qa-1* genes are known to encode, respectively, the catabolic dehydroquinase (Rines, 1969) and a

Fig. 3. Thermal denaturation at 45 °C of QDH and SDH activities in extracts prepared from wild-type (74A) cells induced on: (a) 0.3 % quinate minimal medium; (b) 0.3 % shikimate minimal medium. ●, QDH; ○, SDH.

protein which functions positively in regulating the synthesis of the three catabolic enzymes (Valone *et al.* 1971; Case, 1972; Partridge *et al.* 1972). Although *qa-4* is presumed to be the structural gene for dehydroshikimate dehydrase, this has not yet been unequivocally demonstrated. The *qa-3* locus encodes a single NAD-linked dehydrogenase which recognizes both quinate and shikimate as substrates. *Neurospora* is not the only organism which employs a single enzyme to catalyse the oxidation of these two hydroaromatic compounds. In *Acinetobacter calco-aceticus* the dehydrogenation of quinate and shikimate is catalysed by a single particle-associated enzyme which functions independently of pyridine nucleotides (Tresguerres, De Torriontegui, Ingledew & Canovas, 1970; Tresguerres, De Torriontegui & Canovas, 1970). A similar situation may also exist in *Aspergillus niger* (Cain, 1972).

The clustering of the *qa-2*, *qa-3* and *qa-4* genes and the observation that mutant alleles of these genes complemented rapidly with *qa-1^r* and slowly with *qa-1^s* strains suggested that

Fig. 4. Thermal denaturation at 43 and 45 °C of QDH and SDH activities in an extract prepared from an *arom-1* (1183) strain induced on polyaromatic supplemented 0.3 % quinate medium. Incubated at 43 °C: ■, QDH; □, SDH. Incubated at 45 °C: ●, QDH; ○, SDH.

these three genes may be transcribed as a single mRNA which is initiated at one promoter. Polarity appears to be a general characteristic of all operons that are transcribed into polycistronic messenger RNA (Ames & Martin, 1964). The decrease in enzyme production by genes located on the side of the mutated gene distal to the promoter is a consequence of the introduction of a nonsense polypeptide chain-terminating codon (Newton, Beckwith, Zipser & Brenner, 1965; Newton, 1966; Martin, Whitfield, Berkowitz & Voll, 1966; Imamoto, Ito & Yanofsky, 1966). Because nonsense mutants act at the level of translation (Brenner & Stretton, 1965; Garen, 1968), it is generally assumed that the isolation of pleiotropic mutants and the demonstration that these are suppressible by known suppressors of nonsense triplets constitutes proof of a polycistronic mRNA. Therefore, to determine if the *qa* cluster is transcribed as a single messenger, attempts were made to recover pleiotropic mutants. One doubly deficient strain, RR228A, was recovered from among 250 isolates obtained as 'rever-

Fig. 5. Thermal denaturation at 37 °C of QDH and SDH activities in extracts prepared from wild type (74A) and a temperature-sensitive *qa-3* revertant (M16-1A-RV3) induced on 0.3% quinate medium. Wild type: ●, QDH; ○, SDH. M16-1A-RV3: ■, QDH; □, SDH. Mixture: ▲, QDH; △, SDH. The protein concentration was 15.4 mg/ml in the wild-type extract and 13.8 mg/ml in the M16-1A-RV3 extract.

tants' of an *arom-1* allele. Several other mutants were found to have reduced levels of catabolic dehydroquinase. Since nonsense mutants are recovered at a much higher frequency in other fungal systems (Fink, 1966; Giles *et al.* 1967*a*; Case & Giles, 1968), the recovery of only one mutant lacking the two catabolic activities suggests that RR228A may not be a polar nonsense mutant. If the three genes are transcribed independently, RR228A may contain either a deletion which extends through the *qa-2* and *qa-4* regions, or two independent mutational events. If this is the case, the reduced levels of catabolic dehydroquinase in other mutant isolates (RR53A, RR70-B23-1A and RR134-B36-1A) indicate that synthesis of QDH and dehydroquinase is not coordinate. A lack of coordinancy may also explain the enzyme levels observed in strain RR231A, which has reduced levels of dehydroquinase and very low

levels of QDH and PCA oxygenase. However, a nonsense mutation in the distal region of the *qa-3* locus could also produce these results. Another interpretation compatible with transcription via a single mRNA is also possible. The *qa-1* and *qa-3* genes encode enzymes essential to the revertant phenotype. If either of these genes is located between the *qa-2* and *qa-4* loci, a loss of both catabolic dehydroquinase and DHS dehydrase could result only from separate mutational events. These explanations are consistent with the low frequency with which double deficient mutants are recovered.

A consideration of other systems supports the possibility that the *qa-2*, *qa-3* and *qa-4* genes are transcribed as a single mRNA. Nonsense mutations in bacterial operons result in reduced translation of the genes on the operator-distal side of the mutated gene, but do not abolish synthesis of these enzymes. The degree of polarity created by nonsense codons depends upon the location of the mutation within the structural gene (Ames & Hartman, 1963; Newton *et al.* 1965; Newton, 1966; Fink & Martin, 1967), and upon the efficiency of the subsequent chain initiator region in phase correction (Martin *et al.* 1966). Unlike the bacterial operons, nonsense mutations in the *his4* region in yeast and the *arom* cluster in *Neurospora* show complete polarity (Fink, 1966; Shaffer, Edelstein & Fink, 1972; Giles *et al.* 1967a; Case & Giles, 1968). It has been suggested that the *his4* region of yeast may encode a single multifunctional polypeptide chain (Shaffer *et al.* 1972). This may also be true of the *arom* gene cluster in *Neurospora* (Gross, 1969), although present evidence indicates that the final multi-enzyme complex can be dissociated relatively easily into smaller components (Jacobson, Hart, Doy & Giles, 1972). In this regard, these two systems apparently differ from the *qa* cluster, since the first three enzymes of quinate-shikimate catabolism do not remain associated during extraction (Rines, 1969). Thus, the apparent absence of an enzyme aggregate and the low frequency of absolute polar mutants may suggest that the *qa* cluster is organized like a bacterial operon: the three polypeptides are synthesized sequentially from a common messenger containing internal translation start signals. The three clustered structural genes which specify the galactose enzymes in yeast (Douglas & Hawthorne, 1964; Bassel & Mortimer, 1971) may be organized similarly, since no evidence yet exists for polarity mutations in that cluster. According to this hypothesis, the strains with reduced levels of catabolic dehydroquinase may be explained as partial polar mutants. The recovery of only one absolute polar mutant may indicate that the three translation initiation regions are extremely efficient. Evidence now exists which suggests that the early genes of bacteriophage T7, for which no polar mutants are known, are transcribed as a polycistronic mRNA which is cleaved into monocistronic messengers by a specific endonuclease before or concomitantly with translation (Dunn & Studier, 1973). A demonstration that the pleiotropic phenotypes exhibited by several *qa* mutants are caused by nonsense mutation must, however, await proof that these mutants are suppressible.

The failure of the *qa-1^F* and *qa-3* mutants to complement on shikimate medium initially suggested that these loci may not be distinct. However, the demonstration that a *qa-1^F* mutant encodes an intact SDH enzyme and that *qa-3* mutants possess a wild-type regulatory protein (Table 2) established the functional independence of the two loci.

Growth at the expense of shikimate may be dependent upon its conversion to DHS, which functions as the true inducer, by trace levels of SDH. Thus, before induction in the presence of shikimate, the intracellular concentration of DHS is dependent upon the basal levels of SDH. A certain minimum concentration of inducer, which is determined by the affinity of the inducer for the regulatory protein and the number of regulatory molecules in the cell, is required to promote enzyme synthesis (Herzenberg, 1959; Chaleff, 1974). It is proposed that the wild-type strain possesses sufficient basal SDH activity to generate

enough DHS to effect induction. Once induction commences, the process becomes autocatalytic: the synthesis of enzyme is accompanied by a higher rate of conversion of shikimate to DHS which results in increased induction. If the *qa-1*⁺ gene product is not present in excess (Cove, 1969) and if a gene dosage effect is assumed in the heterokaryon (Valone *et al.* 1971), a (*qa-1*⁺ *qa-3*) + (*qa-1* *qa-3*⁺) heterokaryon should possess only 25 % of the wild-type basal levels of SDH (assuming a 1:1 nuclear ratio). It is possible that in this circumstance SDH levels are too low (if, indeed, any SDH at all is synthesized in this strain in the absence of inducer) to generate sufficient DHS to promote induction. This hypothesis predicts that a heterokaryon heterozygous for *qa-1* and *qa-3* alleles should be able to grow on shikimate medium following pre-induction on quinate medium.

I thank Dr N. H. Giles for support, valuable discussions, and criticism of the manuscript, and Drs M. Case, C. Partridge, M. Berlyn, R. Gravel and P. Carlson for their helpful contributions and encouragement.

This research was supported by Public Health Service Genetics Training Grant GM 397 and by Atomic Energy Commission Contract AT (30-1)-3098, both administered by Dr N. H. Giles.

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