MUTANTS IN THE *arom* GENE CLUSTER OF *NEUROSPORA CRASSA* SPECIFIC FOR BIOSYNTHETIC DEHYDROQUINASE[1]

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Received August 7, 1968

IN *Neurospora crassa* a gene cluster encodes a multienzyme aggregate of five enzymes catalyzing steps two through six in the pre-chorismic acid part (Figure 1) of the aromatic synthetic pathway (GILES, CASE, PARTRIDGE, and AHMED 1967a). Two distinct categories of mutants have been detected within this *arom* cluster: single gene mutants which lack only one of the activities and pleiotropic ("polarity") mutants which lack two or more activities (Figure 2). A major class of pleiotropic mutants consists of non-complementing types which lack all five activities, map within the proximal (*arom-2*—DHQ synthetase) structural gene in the cluster, and are (in at least some cases) suppressed by nonsense suppressors (CASE and GILES 1968). These mutants provide the major evidence that the *arom* cluster is normally transcribed in a polarized fashion as a single m-RNA which is subsequently translated also in a polarized fashion into the multienzyme aggregate.

Previous studies failed to yield mutants lacking only dehydroquinase (DHQase), despite the fact that the absence of this activity in many non-complementing mutants indicated that the *arom* cluster encodes the DHQase. Subsequently, evidence was obtained that Neurospora has two different DHQases (GILES, PARTRIDGE, AHMED, and CASE 1967b). One of these is a catabolic enzyme induced by quinic acid (QA) or dehydroquinic acid (DHQ). The other, which is constitutive and associated with the *arom* aggregate, is the biosynthetic enzyme. The hypothesis was proposed that single gene mutants in the *arom* cluster lacking the synthetic activity were not recovered because such mutants would accumulate DHQ, induce the catabolic enzyme, and thus exhibit no aromatic requirement. Proof of this hypothesis required the development of a procedure for isolating mutants in this presumptive structural gene in the *arom* cluster encoding the synthetic DHQase. The present paper describes such a procedure and discusses the genetical and biochemical properties of mutants which have been obtained in the predicted category.

MATERIALS AND METHODS

Origin and nature of the qa-1 *mutant:* Wild-type and all *arom* mutant strains studied previously had shown the appearance of high levels of inducible DHQase activity whenever QA

[1] These studies were supported in part under Contract AT(30-1)-3098 with the Atomic Energy Commission. One of the authors (H.W.R.) holds a pre-doctoral fellowship from the National Institutes of Health.

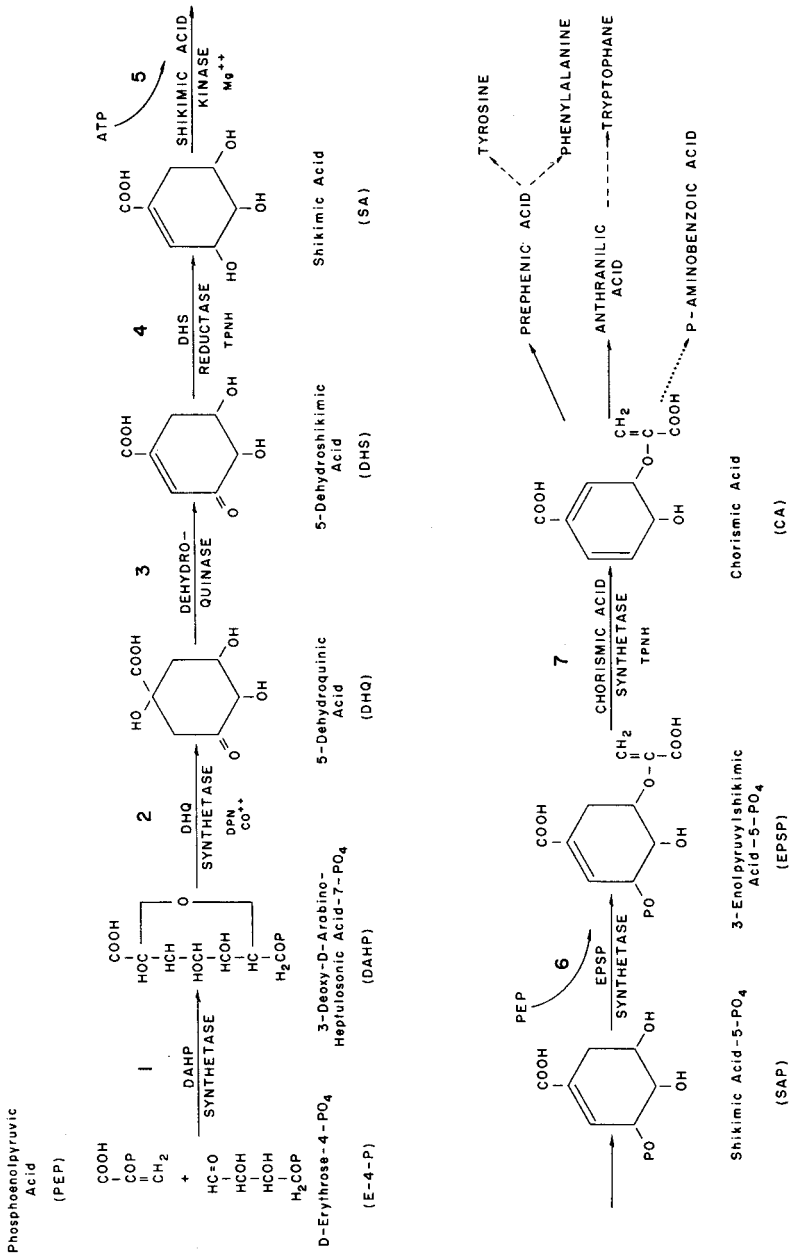

FIGURE 1.—Reactions in the biosynthesis of chorismic acid (in the polyaromatic pathway) in *Neurospora crassa*.

was present in the growth medium (GILES *et al.* 1967b). However, when a group of 25 non-complementing *arom* polar mutants was being tested for induction levels, by chance one strain (1065A) was discovered which produced a very low level of the inducible DHQase in response to the addition of either QA or DHQ to the growth medium. The amount of induced

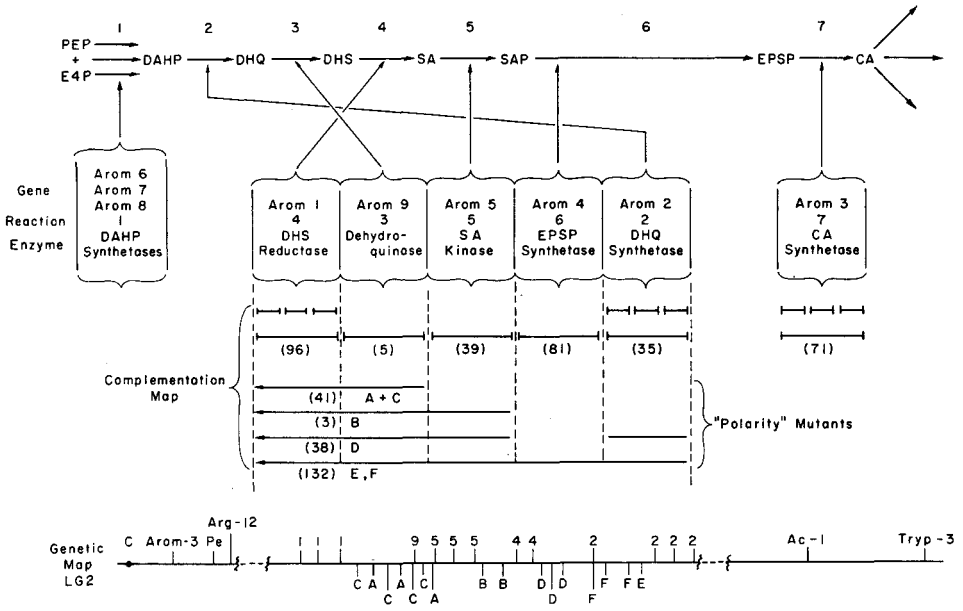

FIGURE 2.—The organization of the *arom* cluster in *Neurospora crassa* on the basis of complementation, biochemical, and genetic recombination data. Reactions in the polyaromatic pathway prior to chorismic acid are indicated at the top of the Figure. Below are shown the structural genes which encode the indicated enzymes catalyzing specific reactions. (The three genes encoding the three DAHP synthetase isozymes and the *arom-3* gene encoding CA synthetase, none of which are within the *arom* cluster, are also included in the Figure.) On the complementation map, numerals in parentheses indicate the number of mutants of a particular type. (The number of *arom-9* mutants indicated is not directly comparable with the numbers of other mutants, since the *arom-9* mutants were recovered in separate experiments—see text.) The short bars on the complementation maps of the *arom-1*, *-2*, and *-3* genes serve only to indicate the occurrence, but not the detailed pattern, of intragenic (allelic) complementation for these mutants. Categories of "polarity" mutants are indicated by the letters A through F. The symbols on the genetic map at the bottom of the Figure, are as follows: L.G. II (linkage group II), C (centromere), *pe* (peach) *Arg-12* (arginine-12), *Ac-1* (acetate-1), *Tryp-3* (tryptophan-3). On the genetic map the approximate positions of single gene mutants within the *arom* cluster are indicated above the line, those of "polarity" mutants, below.

DHQase produced was less than 0.5% of that found in other strains grown under the same conditions. Furthermore, this strain differed from all other *arom* strains tested in being unable to utilize QA (0.3%) as a sole source of carbon for growth.

The failure of strain 1065A to produce inducible DHQase activity resulted from a mutation in a gene independent of the *arom* cluster, as was shown by crossing strain 1065A to wild-type 74a. The new mutant, designated *qa-1* (since the strain fails to grow on QA), segregates independently of the *arom* region (H. RINES, unpublished). Strains containing only the *qa-1* mutation appear not to be affected in aromatic biosynthesis since they grow readily on minimal medium plus sucrose. Also, they have been found by enzymic tests to contain essentially wild-type levels of the five enzymes encoded in the *arom* cluster including the synthetic DHQase. The nature of the genetic lesion which prevents growth on QA is the object of another study presently under way. Meanwhile, the failure of the *qa-1* strain to produce appreciable inducible DHQase activity has permitted the use of this strain to recover mutants lacking the synthetic DHQase activity.

Origin and identification of arom *mutants induced in the* qa-1 *strain:* Two *qa-1* strains were used in experiments to obtain mutants lacking the synthetic DHQase activity. These strains, 1065–7A (*qa-1 pan-2*) and A4–18A (*qa-1 pan-2*), were isolated from crosses to WT (74a) of both the original non-inducible *arom* mutant 1065A (containing a *pan-2* marker) and an F_1 isolate. Conidia of the two strains were treated with ultraviolet (GILES 1951), and in Experiment Y325 conidia of strain A4–18A were also treated with ethylmethanesulfonate (EMS). The standard procedure for EMS treatment was as follows: to a suspension of 2×10^7 conidia in 10 ml of 0.1 M KPO_4 buffer, pH 8, 0.1 ml of EMS was added and the suspension incubated for five hours at 25°C; treatment was terminated by addition of a 6% solution of sodium thiosulfate followed by centrifugation. The conidia were incubated in liquid minimal medium and filtered periodically to remove growing conidia. Following filtration, the remaining conidia were plated onto either Fries minimal medium supplemented with shikimic acid (200 μg/ml), phenylalanine (80 μg/ml), tyrosine (80 μg/ml), and calcium pantothenate (2 μg/ml) (a medium which will support the growth of all mutant types blocked prior to shikimic acid), or onto Fries minimal medium containing a complete aromatic supplement (GILES *et al.* 1967a) plus calcium pantothenate. All polyaromatic auxotrophic mutants recovered were identified by complementation tests with appropriate *arom* tester strains.

Genetic analyses: Crosses and ascospore platings designed to localize mutants defective for the synthetic DHQase were performed as described previously (GILES *et al.* 1967a). All such crosses carried the *qa-1* mutation in both parents.

Biochemical studies: Assays for the five enzyme activities encoded in the *arom* cluster and studies of the behavior of these activities in sucrose gradients containing either Tris or phosphate buffer were carried out as described previously (GILES *et al.* 1967a,b), with the following modification: all DHQase assay mixtures contained 0.1 M KPO_4, pH 7.4 and 2 mM DHQ (prepared by the method of HASLAN, HAWORTH, and KNOWLES, 1963), plus 0.2 mM EDTA and 1/30 volume of enzyme.

RESULTS

Recovery and preliminary identification of mutants (arom-9) *defective for synthetic DHQase:* In the filtration-concentration experiments employing *qa-1* strains, 196 polyaromatic auxotrophic mutants were recovered. In the first experiment, employing strain 1065-7A (Table 1), only shikimic acid-utilizing mutants were tested for their complementation responses with three tester strains—an *arom-1*, an *arom-2*, and the original non-complementing (polar), non-inducible *qa-1 arom* strain 1065. This procedure was used with the expectation that mutants defective for the synthetic DHQase would be able to utilize shikimic acid for growth. In this experiment, one mutant (A4A) was recovered which gave positive complementation responses with the *arom-1* and *arom-2* testers, but was negative

TABLE 1

Categories of arom *mutants recovered in filtration-concentration experiments with non-inducible* qa-1 *strains. See text for further details*

Strain	Arom mutant types recovered								
	arom-1	*arom-9*	*arom-5*	*arom-4*	*arom-2*	A or C	D	E or F	*arom-3*
1065–7A*	2	1	.	.	2	.	.	.	.
A4–18A	7	4	10	10	11	7	11	67	66

* Only those mutants growing on shikimic acid were classified by complementation tests.

with the polar mutant 1065. The preliminary conclusion that this mutant was defective for the synthetic DHQase has been confirmed by enzyme assays which will be discussed later. This category of mutants has been designated *arom-9*, since the designations *arom-6*, *-7*, and *-8* have been employed by Doy (1968) for the three structural genes encoding the three DAHP synthetase isozymes.

In a second more extensive experiment utilizing strain A4–18A (*qa-1*), four additional *arom-9* mutants were recovered (Table 1). In this experiment all categories of *arom* mutants were identified by complementation tests employing all six types of presumptive single gene mutants, including the first *arom-9* mutant (A4A), plus appropriate pleiotropic *arom* cluster testers. The results indicate that in this sample *arom-9* mutants were recovered with about one half the frequency of the other four single gene mutants types in the *arom* cluster. As in previous experiments, the types most frequently recovered were *arom-3* (chorismic acid synthetase) mutants and non-complementing polar (E and F) mutants in the *arom* cluster.

The five available *arom-9* mutants have been tested for complementation in all possible pairwise combinations with negative results.

Genetic localization of arom-9 *mutants:* Crosses of the *arom-9 qa-1* double mutants by wild type showed that the two mutations segregated independently. As expected, strains carrying the *arom-9* mutation alone grew well on minimal medium. They were distinguishable from wild type only by biochemical assays, which will be discussed later. Because of the difficulties involved in identifying the *arom-9* trait in the absence of the *qa-1* gene, it was found necessary to introduce the *qa-1* mutation into all strains used in genetic crosses with *arom-9* mutants in order to be able to detect *arom-9* isolates by growth tests on minimal medium.

On the assumption that the *arom-2* mutant sites were within the *arom* cluster, fifteen mutants in the cluster representing all single gene mutant types and all classes of pleiotropic ("polarity") mutants were first crossed to the *qa-1* strain so that double mutants could be isolated. These double mutants (*arom qa-1*) were then crossed with the original *arom-9 qa-1* mutants and ascospore platings were made on minimal medium to determine prototroph frequencies. (The crosses with one mutant, 325-M5, were infertile.) The results of these crosses are summarized in Figure 3. This Figure also includes previously unpublished data from two-point crosses of many different mutants in the *arom* gene cluster. (In crosses between mutants in different single genes, prototrophs arising from recombination can be readily distinguished from pseudo-wild types by the slower initial growth rates of the latter.) These comparative data localize four of the *arom-9* mutants very close to one of the C polar mutant types (1148) which lacks both DHS reductase and synthetic DHQase activities (on the basis of both complementation tests and enzyme assays). The prototroph frequencies based on viable ascospores plated range between 0.5 and 4.5×10^{-6} in these crosses. The results thus locate the *arom-9* structural gene encoding the synthetic DHQase within the *arom* cluster between the *arom-1* gene, which encodes the DHS reductase, and the

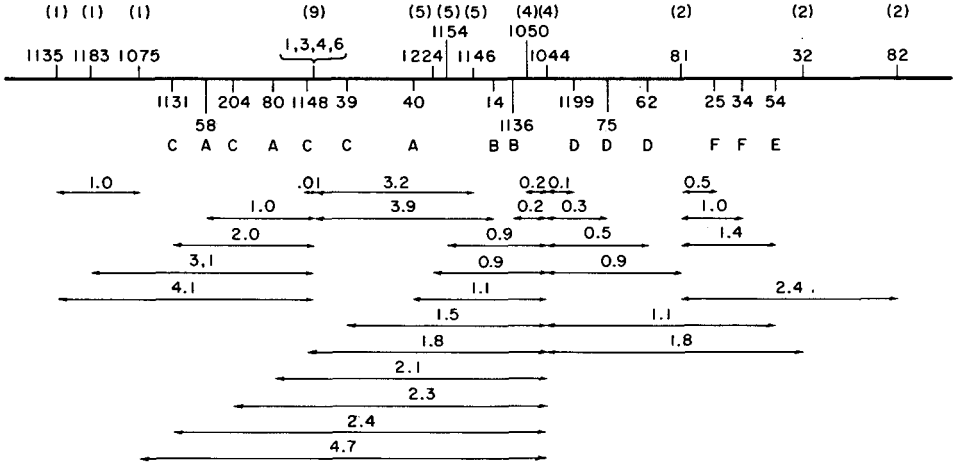

FIGURE 3.—Genetic map of the *arom* gene cluster in *Neurospora crassa*. The single gene mutant types—*arom-1*, *-2*, *-4*, *-5*, and *-9* (identified by the numbers in parentheses above the individual mutant numbers) are indicated above the line. The various types of "polarity" mutants (A through F) are indicated below the line. Distances between mutants are given as prototroph frequencies per 10^4 viable ascospores.

arom-5 gene, which encodes the shikimic acid kinase. Recombination data from crosses between *arom-9* mutants are not available, since to date all crosses between these mutants have been sterile.

Biochemical studies: The five *arom-9* mutants have been studied biochemically both by enzyme assays of partially purified extracts and by sucrose gradients of such extracts. Table 2 presents a comparison of the levels of inducible and synthetic DHQase activities, as well as the levels of the other activities controlled by the *arom* region of *N. crassa*, for the various strains involved in this study. Values are given (1) for the wild-type strain 74A grown in both the presence and absence of QA (500 μg/ml) in the growth medium, (2) for the strain A4-18A, the *qa-1* strain used in the principal mutant hunt for *arom-9* mutants, grown in the presence and absence of QA, and (3) for the five *arom-9* mutants recovered, both with and without the *qa-1* mutation in their genomes, grown in the absence of QA. These data demonstrate that neither the *arom-9* nor the *qa-1* mutations have any appreciable effect on the levels of the four other enzymes which together with the synthetic DHQase form the *arom* enzyme aggregate encoded in the *arom* gene cluster.

The criterion of thermolability was used to determine whether the DHQase activity present represented the constitutive (thermolabile) or the inducible (thermostable) form (see GILES *et al.* 1967b). As has been stated earlier, strains containing the *qa-1* mutation produce only very low levels of inducible DHQase, even under inducing conditions, but their levels of constitutive activity are essentially normal. When the *arom-9* mutants were tested as *qa-1 arom-9* double mutants (the form in which they were first isolated), only A4A exhibited detectable constitutive DHQase activity, and this represented only about 8% of the specific

TABLE 2

Comparative data on aromatic enzyme specific activities in WT (taken as 100), in a qa-1 *mutant, in* arom-9 qa-1 *double mutants, and in* arom-9 *mutants. See text for further details*

Strain No.	Genotype	Quinic acid (500 μg/ml) in medium	Dehydroquinase		Enzyme activities		SA‡ kinase	EPSP‡ synthetase
			Inducible*	Constitutive	DHS reductase	DHQ synthetase		
74A	W.T. (*qa-1*+)	0	0.0	100	100	100	100	100
74A	W.T. (*qa-1*+)	+	146.0	ca.100	112	. . .	100	69
A4-18A	*arom-9*+ *qa-1*	0	0.7	160	147	. . .	122	156
A4-18A	*arom-9*+ *qa-1*	+	0.8	147	125	. . .	147	156
325-M1	*arom-9 qa-1*	0	0.5	0	107	107	111	100
325-M3	*arom-9 qa-1*	0	0.6	0	106	90	108	107
325-M5	*arom-9 qa-1*	0	0.8	0	88	. .	122	117
325-M6	*arom-9 qa-1*	0	0.6	0	80	87	113	107
A4A	*arom-9 qa-1*	0	0.4	8	90	74	91	97
325-M1-9	*arom-9 qa-1*+†	0	27.0	. . .**	110	97	90	90
325-M3-9	*arom-9 qa-1*+	0	24.0	. . .**	102	87	106	143
325-M5-10	*arom-9 qa-1*+	0	29.0	. . .**	114	97	102	98
325-M6-11	*arom-9 qa-1*+	0	32.3	. . .**	136	115	95	107
A4-11	*arom-9 qa-1*+	0	25.5	. . .**	122	109	117	78

* The inducible dehydroquinase activities are expressed as: mμ moles DHS formed/min/mg protein in 0–50% saturated $(NH_4)_2SO_4$ samples. The inducible form of the activity is defined as that portion of the total which is stable at 71°C for 10 min in 100 mM K phosphate pH 7.4 plus 1 mM EDTA. (cf. GILES *et al.* 1967b).

** Not detectable in the presence of the much higher levels of heat-stable activity.

† *arom-9 qa-1*+ strains grown in the presence of 500 μg/ml quinic acid contain substantially more inducible dehydroquinase.

‡ These two activities were assayed by method (b) (GILES *et al.* 1967a).

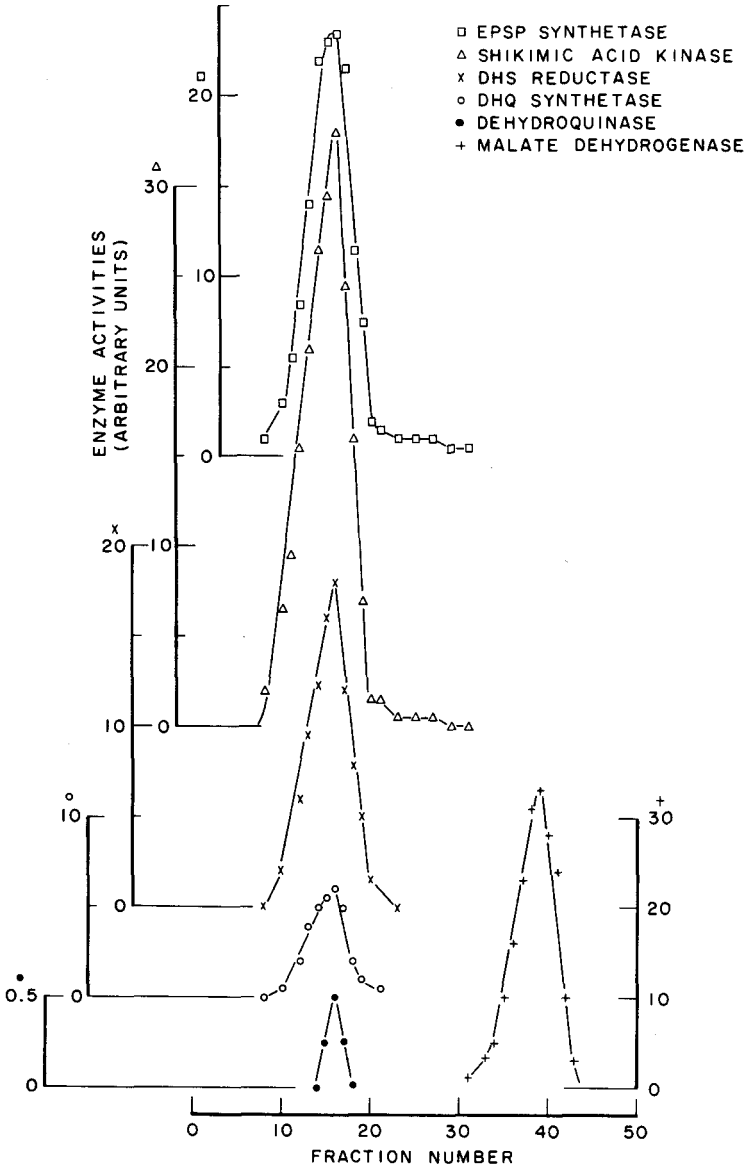

FIGURE 4.—Distribution, after centrifugation in a sucrose density gradient in Tris buffer, of activities of the five aromatic synthetic enzymes encoded in the *arom* gene cluster in *N. crassa* (and of the reference activity—Neurospora malate dehydrogenase) from an *arom-9 qa-1* mutant (A4A). This mutant has a very low constitutive DHQase activity (about 8% of WT). Assays of shikimate kinase and EPSP synthetase were performed by method (b) (GILES *et al.* 1967a).

activity of the original strain from which the mutant was derived (Table 2). Once the *arom-9* mutants were crossed out of the *qa-1* background, even this low level of activity became masked by the presence of the much higher amounts of induc-

ible activity present in the *arom-9* single gene mutants. These levels of inducible activity are similar to those found in many of the *arom* mutants blocked in steps subsequent to the DHQase-catalyzed step and which presumably also accumulate the intermediate, DHQ (GILES *et al.* 1967b; H. RINES unpublished).

Sucrose gradient sedimentation analyses were performed on *arom-9* strains to determine whether either a substantial reduction in, or loss of, the constitutive DHQase activity (which in WT is normally found in an aggregate with the four other activities under the control of the *arom* gene cluster) would affect the integrity of the aggregate, as judged by a change in its sedimentation coefficient. Strain A4A (*arom-9 qa-1*) which contains about 8% of the wild-type synthetic DHQase activity and a second strain (325-M6) which has no detectable synthetic DHQase were used in the initial gradient tests. In both mutant strains all detectable *arom* enzyme activities, including the residual synthetic activity in strain A4A (Figure 4), sedimented together (in either Tris or phosphate buffer) as an aggregate having an *S* value of 11.4, identical with that of the wild-type *arom* aggregate, and slightly less than that of the bovine catalase marker activity employed. This value corresponds to a molecular weight of approximately 220,000. When the "leaky" *arom-9* mutant with the *qa-1* mutation no longer present (A4-11) was run on a similar gradient (Figure 5), the DHQase activity was chiefly in the lighter fractions and had a sedimentation value corresponding to that previously determined for the inducible DHQase (GILES *et al.* 1967b). As shown by assays before and after heating, this activity also possesses the remarkable heat-stability characteristic of the inducible form, whereas the low level of activity in the fractions corresponding to the aggregate position was heat labile, as expected for the constitutive form (Figure 5).

DISCUSSION

The results of the studies described in this paper confirm the hypothesis proposed concerning the organization of the *arom* gene cluster in *Neurospora crassa* (GILES *et al.* 1967a,b). Specifically, the hypothesis explained the absence of a class of mutants lacking only the synthetic DHQase activity by the demonstrated occurrence in *N. crassa* of two DHQases—a constitutive activity in the aromatic synthetic pathway, and an inducible activity in the catabolic pathway. The hypothesis predicted the presence in the *arom* cluster of a structural gene encoding the synthetic (constitutive) DHQase activity. The fortunate discovery of a non-inducible mutant (*qa-1*) which produces very little inducible DHQase, even under conditions which normally lead to the presence of high levels of this activity, has made it possible to select for the expected class of biosynthetic mutants. These mutants (designated *arom-9*) have the expected phenotype, lacking (or having a very low level of) the synthetic DHQase, but possessing (when lacking the mutation, *qa-1*, which prevents induction) high levels of the thermostable inducible DHQase. In addition, these mutants possess essentially normal levels of the other four aromatic synthetic enzyme activities encoded in the *arom* gene cluster, associated in an aggregate having a molecular weight of about

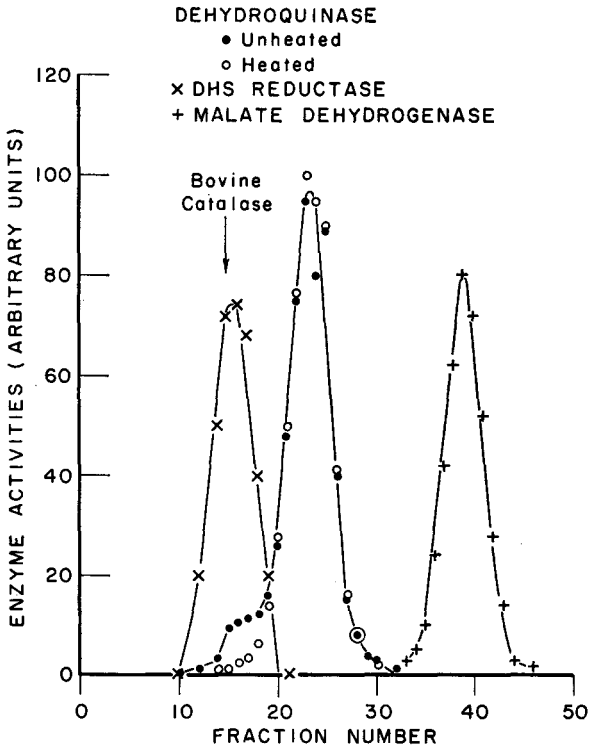

FIGURE 5.—Thermolability tests of the DHQase activities in a sucrose gradient of an extract from an *arom-9 qa-1*+ mutant (A4-11). Assay conditions are described in this paper and in GILES *et al.* 1967b. Exogenous catalase added to the gradient is from beef liver, MW 244,000. The other reference activity is endogenous Neurospora malate dehydrogenase. The position of DHS reductase, a component of the normal *arom* enzyme aggregate, is also indicated.

220,000 (as judged by sedimentation data) indistinguishable from that of the wild-type *arom* aggregate. Recombination data indicate that, as predicted, the *arom-9* mutants map within the *arom* gene cluster, and these data localize the the *arom-9* structural gene between the two structural genes encoding the DHS reductase (*arom-1*) and shikimic acid kinase (*arom-5*). These results require a slight modification (shown in Figure 2) of previous diagrams depicting the presumptive organization of the *arom* cluster (GILES *et al.* 1967a). At that time the relative positions of *arom-1* (DHS reductaseless) mutants and the presumptive synthetic DHQaseless mutants could not be definitely established by complementation data alone, and the latter mutants were tentatively indicated as occupying the most distal region within the cluster.

The overall results strongly support the view that the *arom-9* mutants are the result of missense mutations within the structural gene encoding the synthetic DHQase. The presumptive single amino acid substitutions caused by missense mutations lead to the loss of (or marked reduction in) the synthetic (constitutive) DHQase activity, but do not interfere with the formation of an otherwise appar-

ently normal *arom* aggregate having unimpaired activity levels for the other four aromatic synthetic enzymes normally present.

The present studies have led to a final general understanding of the organization of the *arom* gene cluster in *N. crassa*, as summarized in the diagrams comprising Figures 1 and 2. In addition to confirming predictions concerning the organization of the *arom* cluster, the recovery of mutants lacking activity for the synthetic DHQase will greatly facilitate further investigations of the genetic control of aromatic metabolism, especially in the catabolic pathway of *N. crassa*. The use of an *arom-9* mutant in filtration-concentration experiments has already made possible the recovery of several mutants phenotypically equivalent to the original single *qa-1* mutant, (which has now been shown to be non-inducible for three normally-inducible, sequential enzymes in the catabolic pathway—QA dehydrogenase, DHQase, and DHS dehydrase). Furthermore, additional different mutants have also been recovered, some of which appear to be defective only in the structural gene for the inducible DHQase (H. RINES 1968).

The authors would like to acknowledge the very efficient technical help of MISS BONNIE WOODING.

SUMMARY

In prior experiments, no mutants lacking DHQase only were recovered in *N. crassa* despite evidence that the *arom* gene cluster encodes this activity as part of the *arom* enzyme aggregate in this organism. The absence of these mutants was assumed to result from the demonstrated presence in Neurospora of two DHQases having similar specificities—one the constitutive enzyme in the synthetic pathway, the other an inducible enzyme in the catabolic pathway.—The discovery of a mutant non-inducible for the catabolic DHQase (designated *qa-1*) has made possible the recovery by filtration-concentration of mutants (designated *arom-9*) in the predicted structural gene for the synthetic (constitutive) DHQase. These mutants lack, or have markedly reduced activity for, this enzyme but possess normal levels of the other four enzyme activities encoded in the *arom* cluster. The remaining activities occur in an aggregate having the same molecular weight (as judged by sucrose density gradient sedimentation data) as the wild-type aggregate. The five available *arom-9* mutants do not exhibit allelic complementation when tested in all possible pairwise combinations. The *arom-9* mutants map within the *arom* cluster between the structural genes for shikimic acid kinase (*arom-5*) and DHS reductase (*arom-1*), thus requiring a change in the previously assumed order of the *arom-1* and *arom-9* genes within the cluster. In addition to confirming predictions concerning the general organization of the *arom* cluster, the new *arom-9* mutants have already made possible the recovery of additional new mutants lacking enzyme activities in the inducible catabolic pathway.

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