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STUDIES CONCERNING THE BIOCHEMICAL GENETICS AND PHYSIOLOGY OF ACTIVITY AND ALLOSTERIC INHIBITION MUTANTS OF *NEUROSPORA CRASSA* 3-DEOXY-D-*ARABINO*-HEPTULOSONATE 7-PHOSPHATE SYNTHASE

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SUMMARY

1 Procedures are described for the selection of two classes of presumptive structural gene mutants of the 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (7-phospho-2-keto-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate-phosphorylating), EC 4 1 2 15) (DAHP synthase) of the fungus *Neurospora crassa* (a) Three separate activity-negative mutations have been correlated with isoenzymes inhibited by the end products, tyrosine, phenylalanine and tryptophan, *arom-6* with DAHP synthase (Tyr), *arom-7* with DAHP synthase (Phe) and *arom-8* with DAHP synthase (Trp) (b) Mutants retaining activity but no longer allosterically inhibited (allosteric inhibition-negative) were obtained for each of the three isoenzymes

2. *Arom-6, arom-7* and *arom-8* are unlinked to the "*arom* gene cluster" *Arom-6* maps on linkage group VI L, *arom-7* and *arom-8* both map on linkage group I R but are widely separated Therefore, no DAHP synthase operon or operon-like gene cluster exists In all three cases, the absence of recombination and complementation *in vitro* (enzymic) and *in vivo* (forced heterocaryon) indicates that the two classes of mutations associated with a specific isoenzyme are allelic On this hypothesis a single polypeptide prescribes activity and allosteric inhibition, but the polypeptide is different for each isoenzyme

3 Strains carrying *arom-6* and grown in the presence of phenylalanine, tyrosine and tryptophan have a nutritional requirement for the folic acid precursor 4-aminobenzoate This suggests that DAHP synthase (Tyr) plays a special regulatory role associated with the synthesis of 4-aminobenzoate

4 It is concluded that DAHP synthase is differentially controlled by phenylalanine, tyrosine and tryptophan without channelling of DAHP

Abbreviation DAHP synthase, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase

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INTRODUCTION

In this laboratory the investigation of the 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (7-phospho-2-keto-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate-phosphorylating), EC 4 1.2 15) (DAHP synthase) from the fungus *Neurospora crassa* is aimed at obtaining as complete a description as possible of the nature and role of this important regulatory function (Fig 1) To this end, biochemical observations have been supplemented by genetic analysis, and the results are constantly reviewed in terms of the biology of the organism The present paper describes a biochemical-genetic approach based on isolating mutants of DAHP synthase, on mapping the genes and on using the mutants for biochemical and physiological observations

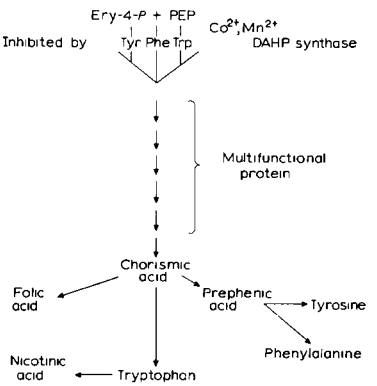


Fig 1 Outline of aromatic biosynthesis in *N crassa* Ery-4-*P*, D-erythrose 4-phosphate, PEP, phosphoenolpyruvate

At the beginning of this work, the inhibition by the three aromatic amino acids had been established[1–3], but the nature of the different portions of enzyme was not known It was anticipated from the known properties[3] and by analogy with *Escherichia coli*[4–6] that differential inhibition was probably based on units of activity capable of existing and functioning as separate isoenzymes This has now been established[4,7–9] in the form of isoenzymes separately inhibited by tyrosine (DAHP synthase (Tyr)), phenylalanine (DAHP synthase (Phe)) and tryptophan (DAHP synthase (Trp)) It was also anticipated that the absence of DAHP synthase mutants implied that multiple genes contributed to the activity and that it was necessary to eliminate more than one of the polypeptide contributions to prevent enzymic function

Some biochemical work with wild type is included in the present paper to provide a criterion under specific conditions The molecular forms of wild-type *N. crassa* 74A and the effects of the substrate phosphoenolpyruvate and the allosteric ligands on them are described in detail separately[9] Some previous reference to mutants of DAHP synthase has been made in a review article and in other papers[4,10,11].

MATERIALS AND METHODS

Organism

Neurospora crassa 74A, a wild-type strain was provided by N Giles and M Case All mutations were introduced into this background Markers used in mapping studies were introduced from stocks having different backgrounds (provided by D G Catcheside)

Mutant isolation

Two classes of presumptive structural gene mutants have been isolated, the selection method employed depended upon the class Mutagenesis was by ultraviolet irradiation

Activity-negative mutants Selection was by filtration enrichment[12] using media supplemented with low levels of protein, with nucleic acid precursors and with traces of vitamins to increase the rate of germination of wild-type conidia and undesirable mutants and so facilitate their removal from the filtration medium

High levels of aromatic amino acids (each 1 mM) were used to inhibit the growth of the desired mutant strain during filtration This inhibition was then counteracted by plating the washed conida on a medium enriched with all three aromatic amino acids (each 1 mM) and 4-aminobenzoate (1 μM) Preliminary characterisation of the isolates was by growth on minimal plates and on plates supplemented with inhibitory levels (1 mM) of the two aromatic amino acids added during filtration Isolates growing on the minimal medium but not on the supplemented medium were assumed to have lost the portion of DAHP synthase controlled by the aromatic amino acid omitted during selection This was confirmed by growth on liquid media followed by assay of a crude dialysed extract for DAHP synthase activity and inhibition properties

Allosteric ligand resistant mutants (allosteric inhibition-negative mutants) These mutants were obtained by a positive selection technique from strains defective in two of the three different activity-negative mutants A selective medium containing the required allosteric ligand (1 mM) inhibits the activity of the remaining isoenzyme so preventing growth of this strain whilst permitting growth of resistant mutants in which the isoenzyme has become insensitive to inhibition

Extracts were prepared from the selected isolates and were assayed for activity in the presence of the previously inhibitory ligand

Growth conditions

Wild type Mycelia were cultured on VOGEL[13] minimal liquid medium N in shaken culture at 25° Mycelial pads were harvested using Buchner filtration and were thoroughly washed with distilled water, each pad was squeezed dry in several thicknesses of tissue, and the wet weight was determined Mycelia required for the extraction of DAHP synthase were immediately frozen and were stored at −10° to −15°

Mutant strains Supplements of the aromatic amino acids (each 1 mM) and 4-aminobenzoate (1 μM) were used for the growth of the triple mutant *(arom-6 arom-7 arom-8)* Single and double mutant strains were grown on the minimal medium Aromatic amino acids (each 1 mM) were used in selective media to inhibit specific isoenzymes as required

Growth tests of arom-6, arom-7 and arom-8 Quadruplicate cultures (100 ml

liquid medium) containing all combinations of phenylalanine, tyrosine and tryptophan (each 1 mM) and the triple supplement *plus* 4-aminobenzoate (1 μM) were used for each strain Duplicate sets were harvested after 36 and 72 h (48 and 84 h *arom-8*) Estimations of the dry weight were made on mycelia dried for 24 h at 80° and cooled in a dessicator

4-Aminobenzoate titration Triplicate cultures (100 ml liquid medium) supplemented with phenylalanine, tyrosine and tryptophan (each 1 mM) over the range of 4-aminobenzoate levels shown in Fig 3 were grown for 48 h at 25° with shaking Estimations of the dry weight were made as previously indicated

Plate tests Plate tests were carried out on solidified medium of VOGEL[13] (1 5% ion agar) using sorbose sucrose in a 5 1 ratio or sorbose glucose fructose in a 40 1 2 ratio to give restricted growth

Crossing techniques Crossing medium was prepared according to WESTERGAARD AND MITCHELL[14] and was supplemented as required Conidia from both parents were inoculated at the same time or in a few cases, where sterility problems existed, the female parent was inoculated and grown for 6 days (25°) prior to the inoculation of the second parent Crosses were maintained at 25° for a minimum of 3 weeks before harvesting

Preparation of forced heterocaryons Forcing markers used included *nic-1*, *arg-1* and *pan-2* in conjunction with the required DAHP synthase mutations Heterocaryons were obtained by placing approximately equal conidial inocula onto minimal agar slopes A heterocaryotic conidial isolate was selected, and the nuclear ratios were determined by testing individual conidia for their nutritional requirements The heterocaryon was grown on the minimal medium and was used for the preparation of extracts for DAHP synthase assays to determine whether complementation *in vivo* had occurred.

Biochemical methods

Extracts Extracts were prepared by grinding 1 part (wet weight) frozen mycelium with 2 parts (volume) 0 05 M KH_2PO_4–NaOH (pH 6 4) and glass powder All the preparation was carried out on ice, and the cell debris was spun down at 22 000 $\times$ *g* for 20 min at approx 4° Dialysis was for a total of 4 5 h against 0 05 M KH_2PO_4–NaOH (pH 6 4) (3 $\times$ 1-l changes) at 4°, the extract was used immediately

DAHP synthase activity The activity of DAHP synthase was assayed using the cyclohexanone extraction procedure[3,15]. Absorbance at 549 mμ was read on a Gilford 300 with digital read out, accuracy was $\pm$ *A* 0 001

In the multiple assays required for the analysis of the column fractions, the reaction mixtures were modified in the interests of economy to give a final concentration of 0 25 mM phosphoenolpyruvate and D-erythrose 4-phosphate, and as required, 50 μM phenylalanine, tyrosine and tryptophan in a 0 25-ml reaction mixture The 0 025-ml fraction sample was measured by pipette These levels of substrates are saturating up to an activity equivalent to $A_{549\ m\mu} = 0\ 7$

Protein Protein was estimated by the method of LOWRY *et al* [16]

Agarose-gel filtration Agarose Beads Bio Gel 0 5 m 10% agarose 100–200 mesh were used in a column (90 cm $\times$ 2 5 cm) equilibrated with 0 05 M KH_2PO_4–NaOH (pH 6 4) *plus* phosphoenolpyruvate, dithiothreitol and $MgSO_4$ (each 0 1 mM) unless otherwise indicated and were maintained at 4° The extract (10 ml) was applied, and

TABLE I

MAPPING OF ACTIVITY-NEGATIVE MUTATIONS

The genetic constitutions of each parental strain are indicated by placing one above and one below the line

Locus(allele No.)	Linkage group	Linkage date	Progeny scored
arom-6 (DH1)	VI L	lys-5 ylo-1; arom-6; 27, 47, 70	300
		ad-8; arom-6 lys-5; 78, 347, 113	319
arom-7 (DH1)	I R	arg-1 his-3; arom-7; 45, 13, 50	220
		arom-7 his-3 ad-3; 2, 24, 44	251
arom-8 (DH8)	I R	soft; nic-1 arom-8; 212, 66, 275	255
		os soft arom-8; 35, 109, 138	210

protein was eluted at a flow rate of 60 ml/h Fractions were collected over 6-min intervals and were maintained at approx 4° until they were analysed for the activity of total DAHP synthase and for inhibition patterns within 30 min of collection Protein estimations were made later

The operating range and sieving characteristics of the column were determined using several commercially purified proteins of known molecular weight catalase

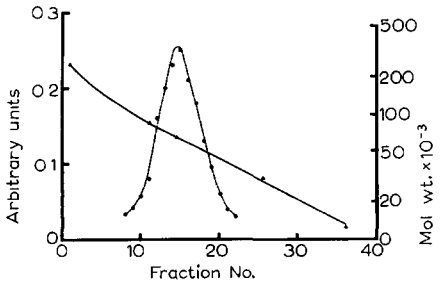


Fig 2 Calibration of an agarose 0 5 M column approx 90 cm × 2 5 cm ▲, standard proteins catalase, (244 000 and 81 000), bovine serum albumin (65 000), deoxyribonuclease (31 000) and ribonuclease (13 000), ●, distribution of bovine serum albumin The arbitrary units are absorbance of colour produced in the protein assay[16] using an E E L colorimeter, OR1 filter

(244 000), bovine serum albumin (65 000), deoxyribonuclease (31 000) and ribonuclease (13 000) (Fig 2) The distribution of bovine serum albumin applied in 10 ml (Fig 2) is typical, and the band width is that characteristic of a single molecular species under these conditions (see also ref 9) Catalase from two commercial sources (Worthington and Sigma) gave a second peak of activity corresponding to 81 000 (Fig 2) The Worthington data sheet indicates that catalase has 6 subunits, suggesting that 81 000 corresponds to a dissociated molecule of 2 subunits

RESULTS

Selection of two classes of presumptive structural gene mutants

Activity-negative mutants A mutant lacking tyrosine-inhibited activity was first obtained (see MATERIALS AND METHODS), and the mutation was named *arom-6* This was then used to introduce a second mutation resulting in loss of phenylalanine-inhibited activity (selected from the parental *arom-6* by filtration enrichment in the presence of tryptophan) On crossing the double mutant into wild type of opposite mating type (74a), the two mutations were readily separated, suggesting that different genes had been mutated The mutation resulting in a loss of phenylalanine-inhibited activity was named *arom-7* A mutant lacking tryptophan-inhibited DAHP synthase was isolated directly from wild type, and the mutation was provisionally named *arom-8* The three mutations obtained above were used in a crossing programme to obtain all combinations of the mutant loci in single strains In the tables and figures, the genotypes of strains containing these mutations are identified by the locus *(arom)* and allele number Strains with different strain numbers may contain the same mutant alleles but are numbered differently in case the unselected background varies

Allosteric inhibition-negative mutants Strains containing two activity-negative mutations were used to obtain mutants resistant to each allosteric ligand The allele numbers of mutations used in this paper are DH25 (phenylalanine resistant), DH22 (tyrosine resistant) and DH19 (tyrosine resistant) and DH19 (tryptophan resistant)

Genetic analysis of mutants

Gene locations The three presumptive structural genes have been located using three point crosses (Table I, see Fig 7 for loci abbreviations) *Arom-6* is located on linkage group VI L, approx 3 map units distal to *lys-5*, *arom-7* is on linkage group I R, approx 2 map units proximal to *his-3* *Arom-8* is 6–10 map units distal to *soft* which is the most distal marker available on linkage group I R More precise location is therefore difficult This analysis confirms that *arom-6*, *arom-7* and *arom-8* are different genes

The locations of the allosteric inhibition-negative mutations in the three resistant strains were studied by crossing each to an *arom-6*, *arom-7*, *arom-8* strain and by determining the frequency of recombination between the activity-negative locus and the resistance locus Recombinants carrying the double mutation cannot be distinguished, but prototrophic recombinants will produce an active enzyme which is sensitive to inhibition by the specific inhibitory ligand Progeny (200) were examined to give a rough estimate of recombination frequency and to establish that progeny of the two parental types segregated with equal frequency No recombinants were found

When only progeny carrying the wild-type allele of the activity-negative mutation are scored, both the recombinant and parental types can be distinguished. Under these conditions approx 300 progeny must be examined for recombinants in order to establish a recombination frequency of $< 1\%$ (1 map unit)

No recombinants were found in analyses of crosses with three resistant strains The examination of 478 progeny from the cross of the *arom-7, arom-8*, tyrosine resistant strain (Strain 265, see Table IV) with the triple activity-negative mutant excluded a recombination frequency $> 0.62\%$ when $P = 0.05$ and $a = 0$ (see Table VIII 1 of FISHER AND YATES[17]) Similarly, in crosses between strains containing the phenylalanine resistance locus (Strain 273) or the tryptophan resistance locus (Strain 280) with the triple activity-negative mutant, 419 and 400 progeny, respectively, were examined The absence of recombinants in these cases exclude recombination frequencies $> 0.72\%$ and $> 0.76\%$, respectively Therefore the resistance loci are closely linked, possibly allelic with the corresponding activity-negative loci

Complementation in vivo and in vitro Complementation studies *in vivo* and *in vitro* were used in an attempt to determine whether the activity-negative and resistance loci are allelic

Complementation *in vivo* was tested in a forced heterocaryon in which growth could occur without complementation between the different mutant enzymes, but if complementation occurred an analysis of the activity of the resultant enzyme would show partial sensitivity to the specific inhibitor for that isoenzyme For example, if complementation occurred between *arom-6* and a tyrosine inhibition-negative mutant, then part of the enzymic product would be inhibited by tyrosine

Strains were developed (Table II) which incorporated the activity-negative mutations concerned, with a forcing marker and the other two activity-negative mutations The second strain carried the allosteric inhibition-negative mutation in conjunction with a forcing marker and the unrelated activity-negative mutations Such pairs were tested for compatibility directly, as heterocaryons will grow independent of complementation other than between forcing markers The nuclear ratios of the heterocaryons were determined by testing a number of conidial isolates for their growth requirements (Table II)

Mycelia were grown from the homocaryotic strains (supplemented media), and the heterocaryotic strains (minimal medium) and extracts were prepared and assayed for DAHP synthase activity and inhibition properties (Table II) In the homocaryotic strains carrying the resistance mutations, the percentage of inhibition of the active isoenzyme by its specific inhibitory ligand did not exceed 8% (*cf* with 100% inhibition of the single isoenzyme parental strain Table IV), and in some cases it was stimulatory No activity was detectable in the *arom-6 arom-7 arom-8 nic-1* strains The heterocaryotic strains showed no increase in sensitivity to the inhibitor, indicating that complementation had not occurred if proteins were produced from the two mutant nuclei

Complementation studies *in vitro* were also carried out using the same strains Extracts were prepared as usual and were tested singly and after mixing The following treatments were used on both single and mixed extracts (a) Standing in ice for 30 min, (b) freezing and thawing slowly and (c) pretreatment with EDTA (2 5 mM final concentration) prior to mixing, the activity being restored by the addition of $CoCl_2$ to the reaction mixtures[3] No increase in enzyme sensitivity to inhibition was found.

TABLE II

THE PROPERTIES OF DAHP SYNTHASE FROM FORCED HETEROCARYONS BETWEEN STRAINS CARRYING ACTIVITY-NEGATIVE AND ALLOSTERIC INHIBITION-NEGATIVE MUTATIONS AFFECTING THE SAME ISOENZYME

The method for producing forced heterocaryons is described in MATERIALS AND METHODS

Strain	*Loci (allele No.)*	*Inhibition (or stimulation) (%)*	*Protein** (mg/ml)*
arom-6		*Tyrosine*	
240	*arom-6* (DH1) *arom-7* (DH7) *arom-8* (DH8) *nic-1* (3416)	0.0	13.8
241	? (DH22) *arom-7* (DH7) *arom-8* (DH8) *arg-1* (K166)	8.1*	18.8
—	Forced heterocaryon of Strains 240 *plus* 241	4.9*	25.4
	Nuclear ratio 1:4		
arom-7		*Phenylalanine*	
239	*arom-6* (DH1) *arom-7* (DH7) *arom-8* (DH8) *nic-1* (3416)	0.0	13.0
244	*arom-6* (DH1) ? (DH25) *arom-8* (DH8) *pan-2* (Y153M96)	7.5	13.4
—	Forced heterocaryon of Strains 239 *plus* 244	3.7	12.9
	Nuclear ratio 1:2		
arom 8		*Tryptophan*	
238	*arom-6* (DH1) *arom-7* (DH7) *arom-8* (DH8) *nic-1* (3416)	0.0	22.2
247	*arom-6* (DH1) *arom-7* (DH7) ? (DH19) *pan-2* (Y153-M96)	2.0	17.8
	Forced heterocaryon of Strains 238 *plus* 247	1.0*	17.7
	Nuclear ratio 1:4		

* Indicates stimulation not inhibition
** Refers to the extract used to study inhibition

The absence of complementation *in vivo* and *in vitro* in all cases strongly suggests that the corresponding activity-negative and allosteric inhibition-negative mutations are allelic (see DISCUSSION) If *arom-6* (allele No DH1) is allelic with the mutation conferring phenotypic resistance to tyrosine, (allele No DH22) then the locus of DH22 is best described as *arom-6*$^{r(DH22)}$ The analogous mutations then become *arom-7*$^{r(DH25)}$ and *arom-8*$^{r(DH19)}$

Physiological studies of the activity-negative mutants

Growth on liquid media The effect of the various combinations of the aromatic amino acids on the growth of these mutants in liquid media is shown in Table III Duplicate flasks were harvested at two different stages of growth The yield (g dry weight) on minimal medium after 72 h (84 h in the case of *arom-8*) was arbitrarily taken as maximal, although slow growth may occur subsequently, this stage approximates the stationary phase The percentage of the total yield attained after 36 h (Table III) clearly reflects the inhibitory effects of the amino acids upon the remaining isoenzymes The combination of the two amino acids inhibiting the remaining isoenzymes is capable of preventing growth for up to 72 h (48 h in the case of *arom-8*)

The 4-aminobenzoate requirement of arom-6 mutants One point of interest is the

TABLE III

RELATIVE GROWTH OF ACTIVITY-NEGATIVE MUTANTS ON VARIOUSLY SUPPLEMENTED MEDIA

Duplicate cultures were harvested in exponential phase (36 h for *arom-6* and *arom-7*, 48 h for *arom-8*)

*Supplement***	*Yield** (%)*		
Loci (allele No.)	*arom-6 (DH1)*	*arom-7 (DH7)*	*arom-8 (DH8)*
Minimal	32	44	19
Phe	Trace	20	12
Tyr	5	Trace	18
Trp	1	5	12
Phe + Tyr	16	42	Trace
Phe + Trp	Trace	10	15
Tyr + Trp	10	Trace	22
Phe + Tyr + Trp	Trace	22	18
Phe + Tyr + Trp + 4-aminobenzoate	20	47	23

* 100% equals yield obtained on minimal medium at 72 h (84 h for *arom-8*)

** Supplements each 1 mM, 4-aminobenzoate 1 μM Trp is a partial inhibitor of the growth of 74 A and derivatives which affects above

marked inhibition of growth of the *arom-6* mutant in the presence of phenylalanine, tyrosine and tryptophan (each 1 mM) This is alleviated by the addition of 4-aminobenzoate (1 μM) This effect is not seen in *arom-7* or *arom-8* Inhibition by phenylalanine, tyrosine and tryptophan was observed with any strain containing the *arom*-6 mutation (listed in Table IV) The requirement for 4-aminobenzoate was titrated, and optimal growth occurs at 3–4 μM (Fig 3) A second independent *arom-6* mutant (allele No DH34) has similar properties

Biochemical studies with extracts of mutants

Activity and inhibition properties Dialysed extracts of strains containing various combinations of mutations were examined for relative activity and inhibition properties (Table IV) The inhibition properties of strains with a single mutant locus confirm the loss of activity of the isoenzyme concerned Inhibition by the other two inhibitory ligands sums to approx 100% The inhibition patterns of the multiple mutant strains are consistent with their component loci The relative activity of all mutant strains is less than wild type, but some degree of independent derepression of the isoenzymes occurs, especially in doubly mutant strains in which the physiological

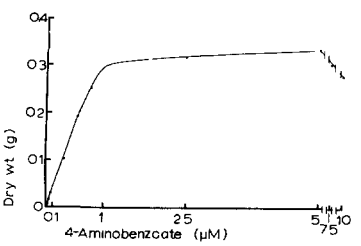


Fig 3 Titration of the 4-aminobenzoate requirement of *arom-6*(DH1) grown in the presence of phenylalanine, tyrosine and tryptophan (each 1 mM)

TABLE IV

DAHP SYNTHASE ACTIVITY AND INHIBITION PROPERTIES IN CRUDE EXTRACTS OF WILD-TYPE AND MUTANT STRAINS

Relative activity is expressed as % of the total wild-type activity Inhibition (%) is relative to the total activity of the particular strains

Strain	*Loci (allele No.)*	*Relative activity*	*Inhibition (or stimulation) (%)*		
			Phe	*Tyr*	*Trp*
74A	wild type	100	47	42	8
73	*arom-6* (DH1)	71	70	4	29
70	*arom-7* (DH7)	89	1*	73	22
100	*arom-8* (DH8)	97	53	36	5*
153	*arom-6* (DH1) *arom-7* (DH7)	19	2	2	97
150	*arom-6* (DH1) *arom-8* (DH8)	51	98	2	4*
152	*arom-7* (DH7) *arom-8* (DH8)	46	4	97	1*
147	*arom-6* (DH1) *arom-7* (DH7) *arom-8* (DH8)	0	0	0	0
265	*arom-6*$^{r(DH22)}$ *arom-7* (DH7) *arom-8* (DH8)	51	0	2	9*
273	*arom-6* (DH1) *arom-7*$^{r(DH25)}$ *arom-8* (DH8)	63	2*	5*	2*
280	*arom-6* (DH1) *arom-7* (DH7) *arom-8*$^{r(DH19)}$	12	2*	3*	2

* Indicates stimulation (%) not inhibition

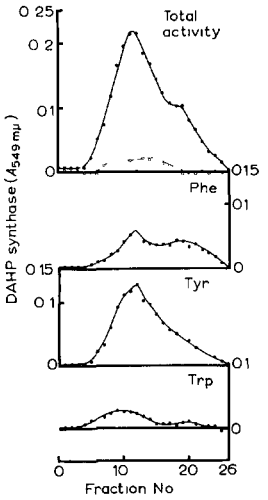
stress on the remaining isoenzyme would be maximal Slight stimulation in the presence of tryptophan occurs in strains containing the *arom-8* mutation

Strains carrying the resistant mutations show activity comparable with that of the double mutant strains from which they were derived, but they are insensitive to inhibition

Molecular sieving. Extracts were examined using a method of molecular sieving on agarose gel beads devised for the separation of DAHP synthase isoenzymes from extracts of wild type[4,8,9] These wild-type isoenzymes inhibited by phenylalanine (DAHP synthase (Phe)), tyrosine (DAHP synthase (Tyr)), and tryptophan (DAHP synthase (Trp)) were also identified in the present work (Fig 4) and were used for comparison with mutant profiles (Fig 5)

The inhibited activities were determined by subtracting activity (as $A_{549\ m\mu}$) measured in the presence of the inhibitor concerned, from activity in the absence of inhibitors The sum of the activities inhibited by the three allosteric ligands was then subtracted from the total activity to determine if any noninhibitable activity remained (see also legend to Fig 4)

Data obtained using molecular sieving of a wild-type extract at pH 6 4 is shown in Fig 4 As the sieving characteristics of the column are known for single species of protein molecules (Fig 2, profile of bovine serum albumin), it is evident that more than one form of enzyme exists in terms of total activity and also the separately inhibited activities DAHP synthases (Phe) and (Tyr) exist in at least two forms which can be distinguished in an elution profile These two peaks possibly represent a range of interacting forms as the peaks are asymmetrical having a trailing edge The major forms will be referred to as higher and lower apparent molecular weight forms according to whether they elute in the earlier or later fractions



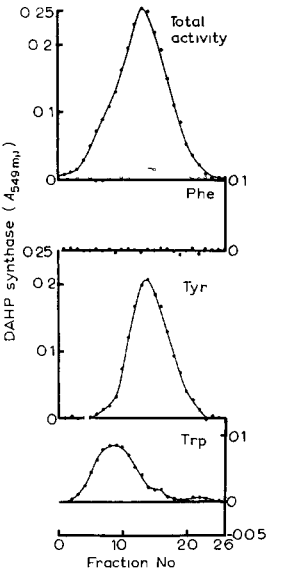


Fig 4 Molecular sieving of wild-type DAHP synthase at pH 6 4 (see MATERIALS AND METHODS) on a calibrated agarose column (as Fig 2) For ease of comparison, total activity and DAHP synthases (Phe), (Tyr) and (Trp) are plotted separately and notated Phe, Tyr and Trp, respectively (●), noninhibited activity (above line), "cross-reaction" (below line) (○) The protein front is in Fraction o

Fig 5 Molecular sieving of *arom-7*(DH7) Symbols, notation and conditions as for Fig 4

Activity-negative mutants The mutations *arom-6*, *arom-7* and *arom-8* lead to the complete loss of the corresponding isoenzyme(s) This is illustrated for *arom-7* (Fig 5) which has lost DAHP synthase (Phe) Similarly *arom-6* has lost DAHP synthase(s) (Tyr) and *arom-8* has lost DAHP synthase (Trp) These losses are expressed in all combinations and the triple mutant has no detectable activity

In the course of these experiments it was noted that the distribution of the wild-type activity between higher and lower molecular weight forms varied from mycelium to mycelium The profiles of mutants tended to be less complex than the corresponding

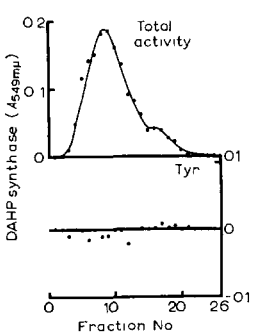


Fig 6 Molecular sieving of *arom-6*$^{r(DH22)}$ *arom-7*(DH7) *arom-8*(DH8) Symbols, notation and conditions as for Fig 4

wild-type isoenzyme(s) Variation within wild-type profiles was eliminated by including a Biogel P-2 column (exclusion limit 1600 molecular weight) prior to the agarose column This removed nonequilibrated small molecules before sieving of protein began and resulted in the isoenzymes appearing in the higher molecular weight forms. In contrast, equilibration of the Biogel P-2–agarose column duplex with phenylalanine, tyrosine and tryptophan (each 10 μM) resulted in DAHP synthases (Phe) and (Tyr) sieving in later fractions (lower apparent molecular weight forms)

Allosteric inhibition-negative mutants Strains carrying mutations producing inactive isoenzymes at two of the loci and a resistant mutation at the third locus were also examined *Arom-6*$^{r(DH22)}$ *arom-7 arom-8* (Fig 6) shows the single peak of activity characteristic of a single isoenzyme, but this is no longer sensitive to inhibition either by its specific inhibitor, tyrosine, or by phenylalanine or tryptophan Similar results were obtained for *arom 6 arom-7*$^{r(DH25)}$ *arom-8* and *arom-6 arom-7 arom-8*$^{r(DH19)}$ The activity distribution of these strains indicates that the higher apparent molecular weight forms are favoured although the P-2 column was not included (see also ref 9).

DISCUSSION

Biochemical genetics

The three new presumptive structural genes, *arom-6*, *arom-7* and *arom-8* are widely separated from each other (Fig 7) and from genes coding for other enzymes of the aromatic path of *Neurospora crassa*. In Fig 7 the approximate locations of these presumptive structural genes have been indicated together with the approximate locations of unrelated markers used in this work Most of the locations are taken from the map published by FINCHAM AND DAY[18], but the position of additional markers was estimated from original papers Distances between genes in map units obtained by different workers are not necessarily the same, and the map of Fig 7 is intended as a summary indicating the gene order on the different linkage groups but not the precise locations

Of particular interest is the finding that the three structural genes of DAHP synthase are removed from the *arom*-gene cluster This latter codes for a multifunctional protein catalysing the five steps of the common pathway immediately following DAHP synthase[19]

Evidence presented in this paper supports the view[4,8,9] that the separately inhibited portions of DAHP synthase can exist as the isoenzymes, DAHP synthases (Tyr), (Phe) and (Trp) *Arom-6* codes for DAHP synthase (Tyr), *arom-7* for DAHP synthase (Phe) and *arom-8* for DAHP synthase (Trp) Further, this study indicated that DAHP synthases (Tyr) and (Phe) can occur in high and low molecular weight forms The change to low molecular weight is favoured by the presence of the aromatic amino acids during sieving, and the high molecular weight forms are favoured when small molecules are separated from crude extracts before the protein mixture is sieved on agarose An elaboration of this approach has shown[9] that the presence of tyrosine is necessary for the conversion to low apparent molecular weight DAHP synthase (Tyr) and similarly phenylalanine for low apparent molecular weight DAHP synthase (Phe) Strains containing the allosteric inhibition-negative mutations, *arom-6*$^{r(DH22)}$ and *arom-7*$^{r(DH25)}$, have DAHP synthase activity that sieves at high apparent molecular weight, although no special steps were taken to remove small molecules from the

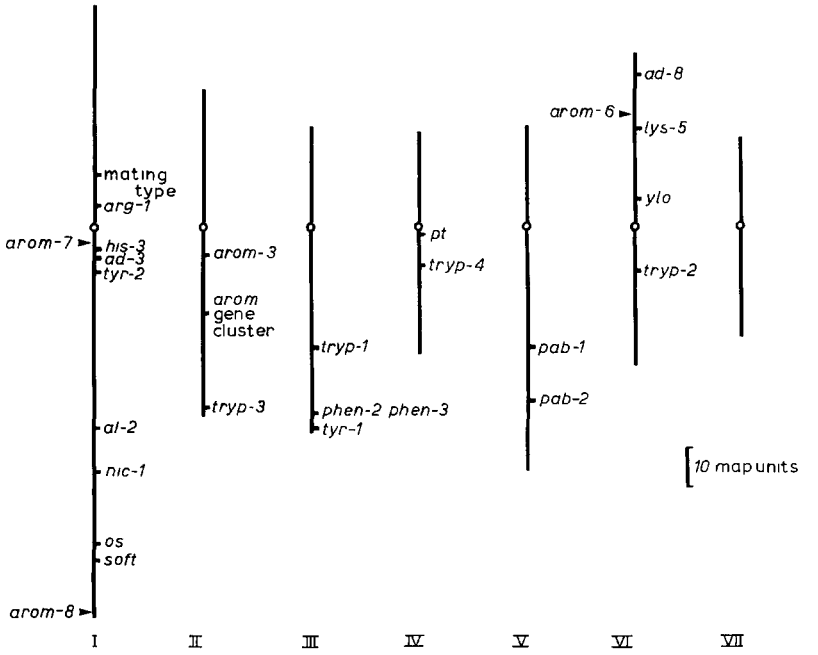


Fig 7 Linkage groups of *Neurospora crassa* showing the approximate locations of the newly mapped genes *arom-6*, *arom-7* and *arom-8* (arrows), other structural genes concerned with aromatic biosynthesis (Fig 1) (*arom* = common path, *tryp* = tryptophan, *tyr* = tyrosine, *phen* = phenylalanine, *pt* = phenylalanine *plus* tyrosine, *pab* = 4-aminobenzoate) and markers used in the present study (*arg* = arginine, *his* = histidine, *ad* = adenine, *al* = albino, *nic* = nicotinic acid, *os* = osmotic, *soft* = soft, *lys* = lysine, *ylo* = yellow) Information concerning the location of these markers was obtained as follows *arom* cluster and *arom-3* (ref 19), *tyr-2* and *tryp-1* (ref 20), *phen-2* and *pt* (ref 21), *phen-3* is allelic with *phen-2* (ref 22), *tryp-4* (ref 23), the remainder (ref 18) Linkage groups are shown according to convention with the left arm uppermost Map distances are only approximately as they tend to vary from one cross to another

environment It is now known[9] that in these strains tyrosine and phenylalanine no longer favour the low apparent molecular weight forms

In all three cases the corresponding allosteric inhibition-negative mutations and activity-negative mutations are probably allelic, that is *arom-6*$^{r(DH22)}$ with *arom-6* (DH1), *arom-7*$^{r(DH25)}$ with *arom-7*(DH7) and *arom-8*$^{r(DH19)}$ with *arom-8*(DH8)

The *arom* gene cluster is regarded as a group of sequential genes and it is stated[19] that complementation *in vivo* and *in vitro* occurs In all three cases of DAHP synthase isoenzyme mutations the absence of recombination and even of partial complementation *in vivo* or *in vitro* leads to the conclusion that the corresponding activity-negative and allosteric inhibition-negative mutations are allelic The data *in vivo* is particularly strong Since the heterocaryon grows without complementation, absence of complementation was decided from the properties of the DAHP synthase present in the heterocaryon Allelism is difficult to prove, and this conclusion is, therefore, provisional Allelism implies that the same cistron, and therefore the same polypeptide, contains the information for activity and allosteric inhibition of DAHP synthase (Phe), for example Similarly, different polypeptides carry this information for DAHP synthases

(Tyr) and (Trp) respectively Since different polypeptides are involved, there is a ready explanation for the separation of isoenzymes

Arguing the alternative case, which assumes that the corresponding activity-negative and allosteric inhibition-negative mutants are nonallelic, then it is necessary to explain noncomplementation Three possibilities can be postulated The first is that all activity-negative mutants are double mutants, the second mutation always being in a gene concerned with the specific allosteric inhibition of that enzyme This is clearly a most improbable situation The second is that the two genes are sequential and that the activity-negative mutations not only prevent the synthesis of the correct polypeptide coded by that gene but also prevent the synthesis of the polypeptide coded for by the gene concerned with allosteric inhibition The improbability here lies in the selection of mutations with identical effects in the three cases examined The third possibility is that the genes are closely linked and that some organisational factor prevents complementation whenever a single nucleus contains one wild-type and one mutant gene

Within the limits of uncertainty indicated by this discussion, it seems reasonable at this stage to designate the mutations as alleles rather than complicate the literature with improbable new *arom* genes

These arguments assume that insensitivity to allosteric inhibition is conferred by mutations affecting the polypeptide carrying the allosteric inhibition recognition site It is possible that in the resistant strains examined the mutations have prevented binding of this polypeptide to the polypeptide primarily concerned with activity

Physiology

The ability of single and double activity-negative mutants to grow on minimal medium means that the utilisation of DAHP is nonchannelled and is available for the synthesis of all the aromatic end products with one possible exception Activity-negative alleles of *arom-6* (affecting DAHP synthase (Tyr)) have a nutritional requirement for 4-aminobenzoate when tyrosine, phenylalanine and tryptophan are added to the medium This did not prevent selection, since 4-aminobenzoate was normally added to the medium with the aromatic amino acids A possible explanation is that the *arom-6* mutations have involved a complete loss of DAHP synthase (Tyr) activity and that the efficiency of inhibition of DAHP synthases (Phe) and (Trp) is sufficient to prevent synthesis of chorismate required for 4-aminobenzoate synthesis DAHP synthase (Tyr) may naturally be incompletely inhibitable as a means of ensuring a supply of 4-aminobenzoate under conditions of strong DAHP synthase inhibition 4-Aminobenzoate is a precursor of the vitamin folic acid and represents a very small percentage of the total aromatic end product requirement (probably $< 0.1\%$) Consequently the first alternative requires very complete inhibition, and an estimate with that accuracy is not possible *in vitro* For *Escherichia coli* it has been suggested that DAHP synthase (Phe) is associated with 4-aminobenzoate synthesis[24] In *Neurospora crassa* this role belongs to DAHP synthase (Tyr) This is not channelling at the level of DAHP synthase or the other common path enzymes since it is the post-chorismate control that actually channels the biosynthetic flow towards folic acid

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