GENE-ENZYME RELATIONSHIPS IN HISTIDINE BIOSYNTHESIS IN *ASPERGILLUS NIDULANS*[1,2]

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ALTHOUGH many nutritional mutants have been isolated and utilized in genetic investigations in *Aspergillus nidulans*, enzymological characterizations of only a few loci have been made (DORN 1965; ROBERTS 1964; FOLEY, GILES and ROBERTS 1965; ROBERTS 1967; HÜTTER and DEMOSS 1967). The present study of histidine mutants undertakes a systematic analysis of gene-enzyme relationships for an entire biosynthetic pathway in Aspergillus. Such extensive correlations in eucaryotic organisms were first established in studies of histidine biosynthesis in *Neurospora crassa* (AHMED 1964) and in *Saccharomyces cerevisiae* (FINK 1964). These studies and the present work have drawn heavily from the biochemical techniques used to elucidate histidine biosynthesis in *Salmonella typhimirium* by AMES and his colleagues (cf. AMES and HARTMAN 1963; SMITH and AMES 1965). The histidine pathway, as determined in Salmonella, is shown in Figure 1.

Most of the loci for histidine enzymes in Neurospora and yeast are widely dispersed, in contrast to the linear contiguity of the loci of the well known histidine operon of Salmonella (AMES and HARTMAN 1963). However, a biochemically heterogeneous continuous genetic region, affecting three of the ten enzymatic activities (reactions 2, 3, and 10 in Figure 1) was found in both these organisms (AHMED, CASE, and GILES 1964; FINK 1966). Each group of mutants defective for only one of the activities occupied a distinct part of the region. A class of noncomplementing mutants defective for all three enzymatic functions was restricted to the terminally located cyclohydrolase (reaction 3) cistron. Another mutant class defective for reactions 2 (pyrophosphohydrolase) and 10 (histidinol dehydrogenase), but demonstrating cyclohydrolase activity in complementation tests, mapped in the central region, among the pyrophosphohydrolase mutants. This polarity in complementation responses and restricted distribution of noncomplementing mutants led the authors to postulate a polycistronic messenger (cf. MARTIN 1963) for this region, which was thus interpreted as a polarized unit of genetic transcription, an operon according to the definition of JACOB and MONOD (1961). In yeast, the suppression of noncomplementing mutants by general (super-) suppressors was interpreted as further evidence that these are

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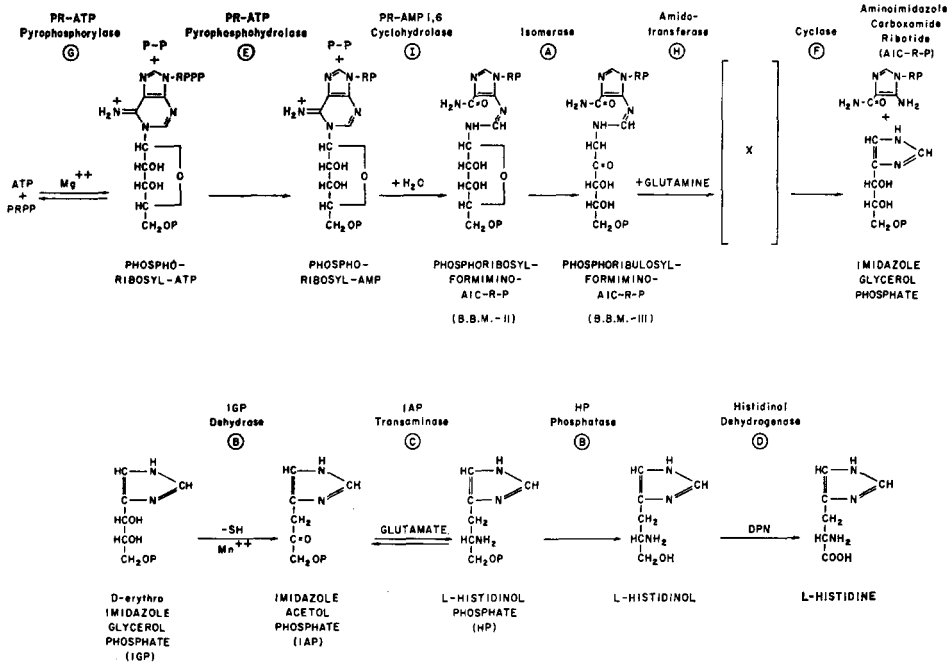

FIGURE 1.—The histidine pathway, as determined in *Salmonella typhimurium* (AMES and HARTMAN, 1963; SMITH and AMES 1965). The encircled letters indicate the Salmonella genes specifying the enzymes. Abbreviations: PRPP, 5-phosphoribosyl-1-pyrophosphate; PR-AMP and PR-ATP, N-1-(5'phosphoribosyl)adenosine mono- and tri-phosphate; BBM, bound Bratton Marshall compounds; and AIC-R-P, 5 amino-1-(5'phospho-ribosyl)-4-imidazolecarboxamide.

"polarity mutations" (cf. AMES and HARTMAN 1963) in an operon (FINK 1966).

It is historically interesting that PONTECORVO (1950) in his early studies of Aspergillus postulated clusters of functionally related genes, but that no cases of clearly functionally distinct groupings were found (ROPER 1960; PONTECORVO 1952). Although such clusters are now well known in bacteria (DEMEREC 1964), they have been clearly demonstrated in eucaryotic microorganisms only in the above-cited studies of histidine biosynthesis in Neurospora and yeast and in studies of aromatic synthesis in Neurospora (GROSS and FEIN 1960; GILES 1965; GILES, CASE and PARTRIDGE 1965) and the galactose pathway in yeast (DOUGLAS and HAWTHORNE 1966). It is important to determine if this type of organization is widespread and can be observed in other organisms as well. The present study renews the search for functionally related clusters in Aspergillus by examining the histidine pathway and the distribution of the genetic loci specifying the enzymes in this pathway.

MATERIALS AND METHODS

Strains: The strains in which the histidine mutants were induced were: A52– *y*, *bi1*; *Acr1*; *ribo2*, *ve*; A148– *y*, *paba1*; *ve;* and *A160– bi1*; *Acr1*, *w1*; *nic8*; *ve*; which were derived from crosses among translocation-free strains obtained from DR. E. KÄFER and cited by FOLEY *et al.*

(1965); and L1– *y*, *bi1*; *s3*; obtained from DR. E. PEES of the University of Leyden and originally derived from Glasgow stocks (PEES, personal communication). Allele symbols are those of KÄFER (1958) and DORN (1967).

Media: The standard Aspergillus minimal medium (PONTECORVO 1953) was used, with Hutner's trace elements solution (EVERSOLE 1956), as suggested by DR. KÄFER, and with appropriate supplementation for nutritional requirements. For recovery of mutants and haploidization products, 200 μg histidine per ml were added; for growth in genetic and biochemical studies, the histidine supplementation was 40 μg per ml. The media also included 25 μg adenine per ml.

Incubation: Cultures were grown at 37°C.

Isolation of mutants: Ultraviolet-irradiated conidial suspensions were inoculated into liquid histidineless media, and prototrophic mycelial growth was removed by filtration (FRIES 1947; WOODWARD, DE ZEEUW, and SRB 1954) or heat treatment (PEES and ELDREDGE 1964). The remaining, ungerminated suspensions were plated on histidine-supplemented media and screened for histidine auxotrophs by replication using damp velveteen (MACKINTOSH and PRITCHARD 1963) or a closely spaced needle replicator (ROBERTS 1959).

Complementation tests: Complementation tests were performed with heterocaryons synthesized by incubating mixed conidial inoculations in drops of completely supplemented medium on plates of histidine-supplemented minimal medium. Hyphal tips were transferred to pairs of plates of minimal media with and without histidine. Second transfers of mycelia were made if growth on the histidineless plate was not observed; conidia from complementing heterocaryons were streaked on supplemented media lacking histidine to check for reversion to histidine independence. Diploid complementation tests were used only in cases of weak growth of heterocaryon transfers.

Mitotic haploidization procedure: The modification of the procedure of FORBES (1959) described by FOLEY *et al.* (1965) was used.

Meiotic analysis: The standard crossing procedure for Aspergillus (PONTECORVO 1953) was followed, and random spore suspensions from individual hybrid perithecia were analyzed.

Growth of mutants and preparation of extracts for accumulation studies: Strains were grown from loops of conidial inocula, in 3-liter Fernbach flasks containing 250 ml minimal medium supplemented with 40 μg histidine per ml, for 2 weeks in stationary culture at 37°C. Both mycelial extracts and culture medium filtrates were analyzed for accumulation of intermediates. The mycelial growth was collected on a cheesecloth filter, rinsed, and squeezed dry, immersed in 15 ml of 70% ethyl alcohol, and boiled or autoclaved for 3 minutes. An additional 15 ml of alcohol was added; the mycelial particles were filtered off and discarded; the alcohol was evaporated by lyophilization; and the final extract was made by collecting the residue in a few drops of 95% ethyl alcohol.

Growth of mutants and preparation of extracts for enzymological studies: Heavy conidial suspensions were inoculated into liquid supplemented minimal medium containing 40 μg histidine/ml, and grown on shakers at 37°C for 24 hours. The mycelia were collected on cheesecloth or sintered glass filters, rinsed with distilled water and 10^{-3} M Tris HCl buffer, pH 7.4, and lyophilized or in some cases frozen and ground in a mortar embedded in dry ice. The lyophilized mycelia were ground to a fine powder and stored in a desiccator at 4°C. Fifty or more milligrams of powder were suspended in 2 ml of the Tris buffer and the suspension was contrifuged at 20,000 *g* for 20 minutes at 4°C. The supernatant was filtered through a 4 × 1 cm column of Sephadex G-25 (Pharmacia, Uppsala, Sweden) to remove small molecules (SMITH and AMES 1964). Extracts were kept at 0°C. Protein determinations were made by the method of LOWRY (LOWRY, ROSEBROUGH, FARR, and RANDALL 1951).

Bound Bratton-Marshall accumulation tests: This assay for diazotizable aryl amines (BRATTON and MARSHALL 1939) was run as described by AMES, MARTIN, and GARRY (1961) on 0.35 ml of the 2-week-old culture filtrate and on 0.1 ml of the mycelial extract before Sephadex filtration.

Imidazole accumulation tests: Ascending, Whatman No. 1 chromatograms of the alcohol extracts run at 25°C in isopropanol:water:ammonia (70:20:10) were air-dried and sprayed with the Pauly reagent and sodium carbonate, as described by AMES and MITCHELL (1952). Neurospora and yeast mutant extracts and L-histidinol were used for standards.

Histininol dehydrogenase assays: The assay described by AMES, HARTMAN, and JACOB (1963, and that of FINK (1966) were used.

PR-ATP pyrophosphorylase assay: The assay developed by AMES *et al.* (1961) with slight modifications by AHMED (1964) was used.

PR-ATP pyrophosphohydrolase and PR-AMP cyclohydrolase assays: These assays, involving enzymic complementation with Salmonella mutants, are described by AHMED *et al.* (1964).

RESULTS

Complementation and biochemical studies: Two hundred and fifty histidine mutants were isolated. Complementation responses among 27 mutants indicated nine complementation groups and a tenth class of mutants (Group X) which fail to complement with three of these groups—I, VIII, and IX. The remainder of the 250 mutants were tested against Group I, VIII, IX, and X mutants only. Twenty-three Group I mutants, one Group VIII mutant, one Group IX mutant, and 22 Group X mutants were obtained. The accumulation characteristics and the enzymatic activities of representatives of the mutant groups are presented in Table 1. The pathway, as indicated by accumulation characteristics and enzyme assays, is the same as that in Salmonella and the locus designations assigned to these groups (Table 1) correspond to loci controlling corresponding steps in Salmonella. The mutant groups characterized by the accumulation of bound Bratton-Marshall compounds were not further differentiated and these mutant groups were arbitrarily assigned the *A*, *H*, and *F* designations of steps 4, 5 and 6 in Salmonella. No phosphatase (reaction 9) mutants were detected. As shown in Table 1, the *E* mutant is defective for reaction 2 (pyrophosphohydrolase), the *I* mutant for reaction 3 (cyclohydrolase), and the *D* mutants for reaction 10 (histidinol dehydrogenase); the mutants which fail to complement either *E*, *I*, or *D* mutants are defective for all three reactions (2, 3 and 10). Observation of occasional spontaneous reversions of the multiply defective types indicated that these were not deletions.

Genetic analysis: The analysis of haploid segregants from diploids between histidine mutants and multiply marked strains is summarized in Table 2. Failure to recover a marker of the tester strain in combination with the histidine gene indicates that the two genes are on the same linkage group. For example, in the diploid between the strain *hisG*28; *bi1; Acr1, w2; nic8* and FORBES' master strain D (MCCULLY and FORBES 1965) *su1ad20, y, ad20; Acr1; ga11; pyro4; facA; s3; nic8; ribo2*, the histidine allele occurs in random combination with all genes except *w2* on Linkage Group II. The one recombinant can be ascribed to the relatively rare, independent occurrence of mitotic crossing over between the homologs. Similarly, these data indicate that *hisA* is on Linkage Group IV, *hisF* on Linkage Group VII, *hisB* on Linkage Group I, and the *hisC*, *hisD*, and *hisH* loci are on Linkage Group VIII.

Crosses among *E*, *I*, *D*, and multiply defective (*EID*) mutants yielded fewer than 5×10^{-4} prototrophic recombinants, indicating that these four classes of mutations occur within a single region on Chromosome VIII. The *hisC* and *hisH* mutants recombined randomly with *hisD* and with each other. Thirteen percent

TABLE 1

Biochemical characteristics and designation of loci

Complementation group	Mutants	Accumulations* BBM	IG	IA	HL	Enzymatic activities† 1	2	3	10	Enzymic defect	Locus designation
I	33, 34, 4, 516, 745, 776	0	0	+	+	0.7	+	+	0	histidinol dehydrogenase (10)	*hisD*
II	28, 5	0	0	0	0	0	+	+	2.1	PR-ATP pyrophosphorylase (1)	*hisG*
III	13	+	0	0	0	0.9	+	+	6.8	isomerase, amidotransferase or cyclase (4, 5, 6)	*hisH*
IV	40, 6	+	0	0	0	—	+	+	2.2		*hisA*
V	30	+	0	0	0	—	+	+	3.7		*hisF*
VI	38	0	0	+	0	1.0	+	+	2.1	imidazolylacetol phosphate transaminase (8)	*hisC*
VII	39, 54	0	+	0	0	0.4	+	+	3.0	imidazolylglycerol phosphate dehydrase (7)	*hisB*
VIII	54	0	0	0	0	—	0	+	3.9	PR-ATP pyrophosphohydrolase (2)	*hisE*
IX	760	0	0	0	0	—	+	0	3.8	PR-AMP-1,6-cyclohydrolase (3)	*hisI*
X	740, 508, 512, 524, 567, 486	0	0	0	0	—	0	0	0	multiple (2, 3, 10)	*hisIED*

* BBM-bound Bratton-Marshall compounds; IG-imidazolylglycerol; IA-imidazolylacetol; HL-histidinol.

† The numbers 1, 2, 3, and 10 indicate the steps in the pathway, as shown in Figure 1. The specific activities given for reaction 1 (Δ_{OD} 290 mμ/min/mg protein) and reaction 10 (Δ_{OD} 340 mμ/min/mg protein) are for the first mutant listed. Formation of AICAR as a result of the enzymatic activity of combined extracts of an Aspergillus mutant and a reaction 2 mutant or a reaction 3 mutant of Salmonella is indicated by +; failure to form AICAR is indicated by 0. A – indicates that no assay was performed.

TABLE 2

*Arrangement of markers relative to histidine genes after mitotic haploidization**

Mutant		Linkage group							
		I	II	III	IV	V	VI	VII	VIII
hisG	'parental'	26	**36**	19	17	13	11	—†	17
	recombinant	11	**1**	16	20	13	17	—	21
hisA	'parental'	18	22	26	**72**	33	37	8	21
	recombinant	35	31	44	**0**	39	35	11	39
hisH	'parental'	11	—‡	—‡	17	9	14	13	**29**
	recombinant	18	—	—	12	17	15	15	**0**
hisF	'parental'	16	13	14	10	13	9	**95**	8
	recombinant	7	10	9	13	10	14	**0**	15
hisB	'parental'	**48**	23	25	23	13	14	0§	15
	recombinant	**0**	25	8	24	21	20	14	24
hisC	'parental'	20	13	25	19	14	23	—†	**38**
	recombinant	21	30	16	22	23	18	—	**2**
hisD	'parental'	49	31	21	51	31	46	14	**84**
	recombinant	51	41	27	49	45	30	36	**1**

* Data were obtained from independent haploid segregants from diploids between histidine mutants and several different multiply marked strains, primarily the master strains of FORBES (MCCULLY and FORBES 1965); for that reason individual markers in each linkage group are not specified. Data in boldface type indicate linkage of the histidine locus to a particular linkage group.
† Diploid was homozygous for *nic8*.
‡ Diploid was homozygous for *Acr1* and carried no Linkage Group III marker.
§ Insufficient supplementation of the recovery medium resulted in failure to recover histidineless and cholineless (Linkage Group VII) mutants; however, recovery of 14 prototrophic recombinants indicated nonlinkage.

recombination was observed between *hisC* and *ribo2* on Linkage Group VIII. Crosses involving histidine mutants and the *ribo2* and *cha* markers on Linkage Group VIII resulted in 39% recombination between *hisH* and *cha*, 51% between *hisH* and *ribo2*, 35% between *ribo2* and both *hisD* and *hisE*, and 40% recombination between *cha* and *hisD*. The location of the *hisIED* region 35 units from *ribo2*, distant from *cha*, is consistent with a 12% recombination value between a *hisD* mutant and *arg3*, observed by DR. C. F. ROBERTS (personal communication).

DISCUSSION

The pathway for histidine biosynthesis is the same in Aspergillus as in *Salmonella typhimurium*. However, the similarity of pathways is not paralleled by a similarity in the function and distribution of the genes specifying the histidine-synthesizing enzymes. In contrast to the single cluster of genes composing the Salmonella histidine operon, the loci in Aspergillus are dispersed among different chromosomes. In Aspergillus seven unlinked loci were found, one of which includes mutants defective for different enzymatic activities. This region includes *E* (pyrophosphohydrolase), *I* (cyclohydrolase), and *D* (dehydrogenase) mutants.

The similarities in the reaction sequence and the differences in the gene-enzyme relationships are also found in Neurospora and in yeast (FINK 1964; AHMED 1964), whereas in *Staphylococcus aureus*, both the pathway and gene-enzyme relationships appear to be essentially the same as in Salmonella (KLOOS

and PATTEE 1965a,b). In the mycelial bacterium *Streptomyces coelicolor*, the pathway is apparently also the same, with five genes located in a cluster and three additional genes at separate sites (RUSSI, CARERE, FRATELLO, and KHOUDOKORMOFF 1966). These relationships are illustrated in Figure 2. Five apparently unlinked groups of histidine-requiring mutants of *Pseudomonas aeruginosa* have very recently been reported (MEE and LEE 1967).

A minor difference between the histidine pathway as observed in Neurospora and yeast and that determined in Salmonella is the finding of only two mutant classes corresponding to the *F*, *A*, and *H* loci of Salmonella. Either a tenth histidine locus remains to be found (despite massive numbers of mutants isolated) or only two enzymes are utilized for these reactions in Neurospora and yeast. In Aspergillus, three groups of mutants at three distinct loci accumulated BBM compounds and showed activity for the early and late steps in the sequence. One of these loci (*hisH*) was represented by only one mutant in about 100 histidine-requiring mutants examined. Further examination of these mutants is necessary before exact correlations can be made for this segment of the pathway.

The similarities among the three fungi are as striking as the differences in the organization of the histidine genes between the bacterium Salmonella and the fungi Neurospora, yeast, and Aspergillus. In the fungi, the histidine loci are widely scattered, with the exception of the 3-cistron cluster which determines three enzyme activities. The enzymes coded for by this complex (*IED*) region in

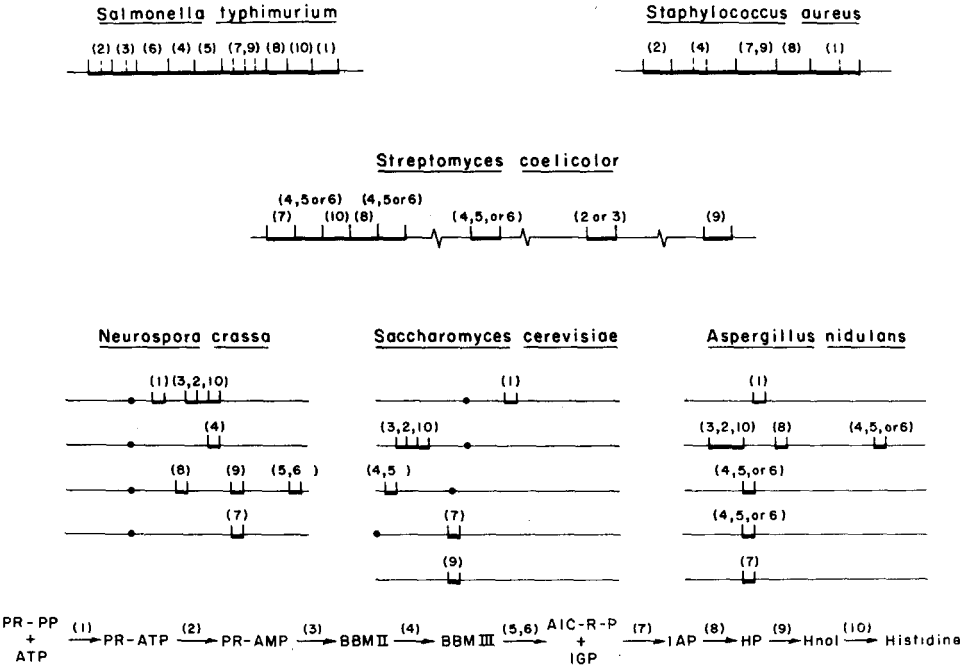

FIGURE 2.—A comparison of the distribution of the histidine loci in six microorganisms. The numbers indicate the steps in the pathway. Abbreviations are explained in Figure 1.

Aspergillus are exactly those specified by the Neurospora (*hist-3*) and yeast (*hi-4*) clusters (AHMED 1964; FINK 1965). In Aspergillus, as in yeast and Neurospora, an unusual class of pleiotropic mutations was found that yielded mutants totally noncomplementing and lacking the three enzyme activities. Functional independence of the *I*, *E*, and *D* activities is indicated by the existence of point mutations which eliminate only one of the activities; functional integration is indicated by the class of mutants lacking all three activities.

The fact that the *I*, *E*, and *D* activities are associated with complex regions in these three ascomycetes suggests that the organization and function of the Aspergillus *IED* region may be analogous to that of the corresponding regions in Neurospora and yeast. The existence of the large class of noncomplementing, multiply defective mutants in the three organisms further suggests an analogous situation. Isolation of more *E* and *I* mutants is required before it can be demonstrated whether the *I*, *E*, and *D* mutants define three distinct subdivisions within this region, and whether the noncomplementing mutants also show a restricted genetic localization. The results of studies with the larger arrays of histidine mutants isolated in Neurospora and yeast would suggest that further mutant isolations in Aspergillus might include an additional mutant class, in which two of the three functions are impaired. These further studies, and studies on the physical state of the gene products, will indicate whether this cluster of distinct but related mutants may indeed be interpreted as a polarized unit of genetic transcription in Aspergillus.

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SUMMARY

Nine classes of histidine-requiring mutants in *Aspergillus nidulans* have been correlated with nine of the ten enzymatic steps in the histidine pathway as determined originally in *Salmonella typhimurium*. Genetic analysis has placed these mutants at seven distinct positions, with one locus in each of the Linkage Groups I, II, IV, and VII, two loci in VIII, and a complex region in Linkage Group VIII. The complex region includes mutants which are defective for the pyrophosphohydrolase, cyclohydrolase, or histidinol dehydrogenase reactions and multiply defective mutants which lack all three of these reactions. These results are similar to those obtained in previous studies with histidine mutants in Neurospora and yeast.

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