GENETICAL AND BIOCHEMICAL STUDIES OF HISTIDINE-REQUIRING MUTANTS OF NEUROSPORA CRASSA. III. CORRESPONDENCE BETWEEN BIOCHEMICAL CHARACTERISTICS AND COMPLEMENTATION MAP POSITION OF *hist-3* MUTANTS[1]

BROOKE B. WEBBER

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

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INTERALLELIC complementation has been demonstrated for mutants at various loci (see review by YANOFSKY and ST. LAWRENCE 1960), including the *hist-3* mutants of Neurospora (WEBBER 1959, 1960; CATCHESIDE 1960). Allelic mutants are tested in various combinations or in all possible combinations of pairs in heterokaryon tests, and the interaction matrix resulting from these tests is used to develop a complementation map. The map thus produced is divided into complementation subunits or complons (DE SERRES 1963). The demonstration of *in vitro* complementation by a number of workers (e.g., WOODWARD 1959; review by CRICK and ORGEL 1964) suggests that complementation results from interaction between defective protein molecules. One might expect that mutants exhibiting different extents or locations on a complementation map would have suffered different types of genetically determined protein alterations.

The *hist-3* mutants of Neurospora were considered allelic on the basis of two criteria: (1) they are closely linked, with a conventional map distance of about 0.03 within the region; (2) when evaluated by heterokaryon tests, all *hist-3* mutants are noncomplementing with a group of *hist-3* mutants which are recovered frequently in forward-mutation experiments. However, on the basis of enzyme assays and tests for accumulation of metabolic intermediates, *hist-3* mutants were assigned (WEBBER 1960) to the following three subgroups: Subgroup 1—mutants which are deficient for histidinol dehydrogenase activity; Subgroup 2—mutants which are deficient for a reaction early in the biosynthetic pathway for histidine; Subgroup 3—mutants which are deficient for the early reaction and histidinol dehydrogenase. Further work by A. AHMED (unpublished) has shown that mutation in the *hist-3* region may affect any or all of three different biochemical reactions in histidine biosynthesis (including histidinol dehydrogenase and two early reactions). The present paper reports the preparation of a complementation map for some *hist-3* mutants and demonstrates that the classification of mutants on the basis of complementation map position corresponds rather precisely with the biochemical subgrouping of these mutants.

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An accompanying paper (WEBBER 1965) reports results of crosses with some of these mutants.

MATERIALS AND METHODS

Tests for allelic complementation: The test tube method of DE SERRES (1962) was used in heterokaryon tests for allelic complementation because it permits the detection of slow or weak heterokaryon formation and growth. *hist-3* mutants were selected by heterokaryon tests with noncomplementary *hist-3* tester mutants (WEBBER and CASE 1960) from a group of heterogeneous histidine-requiring mutants derived by the filtration technique (WOODWARD, DE ZEEUW and SRB 1954) from untreated, X-irradiated, or ultraviolet-(UV)-irradiated 74A wild-type conidia.

A suspension of 5×10^5 macroconidia per ml of Fries' minimal medium was made for each of the mutants to be tested. One ml of a suspension of one mutant was mixed with 1 ml of a second mutant in a 13 × 100 mm test tube to form a bilaterally symmetrical grid containing all possible combinations of pairs. In this way, each pair of mutants is tested in duplicate and each mutant was tested by itself for control growth in triplicate. Tubes were incubated at 22–25°C and observed for mycelial growth and formation of conidia during a 45-day period, although the last positive responses were apparent at 38 days. The late growth responses occurred at essentially the same times in duplicate tubes, and growth was slow, suggesting that these responses did not result from reversion to histidine-independence.

Biochemical tests: Histidinol accumulators (Subgroup 1) were distinguished from nonaccumulators by testing growth media or mycelia with the diazotized sulfanilic reagent (PAULY 1904) as previously described (WEBBER 1960). Subgroup 2 mutants are distinguished from Subgroup 3 mutants by determining histidinol dehydrogenase activity in mycelial extracts (AMES, GARRY and HERZENBERG 1960). For the assays, pads of Neurospora mycelium were grown from inocula of conidia for 5 days at 25°C in stationary 250-ml Erlenmeyer flasks, each containing 50 ml of Fries' liquid medium supplemented with 37.5 μg/ml of L-histidine-HCl-H_2O. The mycelial pads were drained briefly over a vacuum filter, frozen on dry ice, and stored at −20°C prior to use. These pads were thawed and then subjected to vibration for 1 minute at 0°C in a Nossal shaker to break the mycelium. The shaker tube contained about 1 g of mycelium; 3 ml of 0.05 M potassium phosphate buffer, pH 7.5; and about 2 g of acid-washed glass beads (Superbrite, type 090–5005, Minnesota Minning and Manufacturing Company). The Nossal homogenate was then centrifuged at 25,000 × *g* for 15 to 20 min, and the resulting supernatant was subsequently dialyzed against two volumes of potassium phosphate buffer, pH 7.5, for a total of 4 to 5 hours. The preparations usually contained 3 to 8 mg/ml of protein as determined by the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951).

The histidinol dehydrogenase reaction is: L-histidinol + 2DPN+ + H_2O ⟶ L-histidine + 2DPNH + 2H+. The assay procedure utilizes the colorimetric observation of diaphorase-catalyzed reduction and bleaching of 2,6-dichlorophenol indophenol by the DPNH formed in the above reaction. The assay cuvette contained: (1) 2.9 ml of dichlorophenol indophenol buffer mix (2.5 ml of a 1 mM solution of 2,6-dichlorophenol indophenol; 45 ml of 0.2 M triethanolamine-HCl buffer, pH 8.4; and 47.5 ml of water); (2) 0.05 ml of a 0.050 M solution of DPN+; (3) 0.01 ml of diaphorase solution composed of 20 mg of diaphorase (from Worthington Biochemical Corporation) dissolved in 5 ml of 0.01 M triethanolamine-HCl buffer, pH 7.5; (4) 0.05 to 0.5 ml of Neurospora extract. This mixture was incubated to exhaust substrates for other dehydrogenases, and the absorption at 600 mμ (versus at water blank) was observed with a Beckman DU spectrophotometer until no further dye reduction was observed (usually 1 to 2 minutes). Specific enzymic activity was assayed by readings at 30-second intervals after the addition of 0.02 ml of a 0.1 M L-histidinol solution. The rate is linear for the first 2 to 3 minutes and proportional to the amount of Neurospora extract added to the reaction mixture. One unit of activity is defined as 0.1 μmole of dye reduced per hour under these assay conditions. Typically two concentrations of extract were measured in the enzymic reaction, namely 0.1 ml and then 0.05, 0.2, or 0.5 ml depending upon the activity observed with the 0.1-ml sample.

Two types of alterations were made in the procedure of AMES *et al.* (1960). Dye was reduced to a concentration which could be observed readily with the Beckman DU spectrophotometer

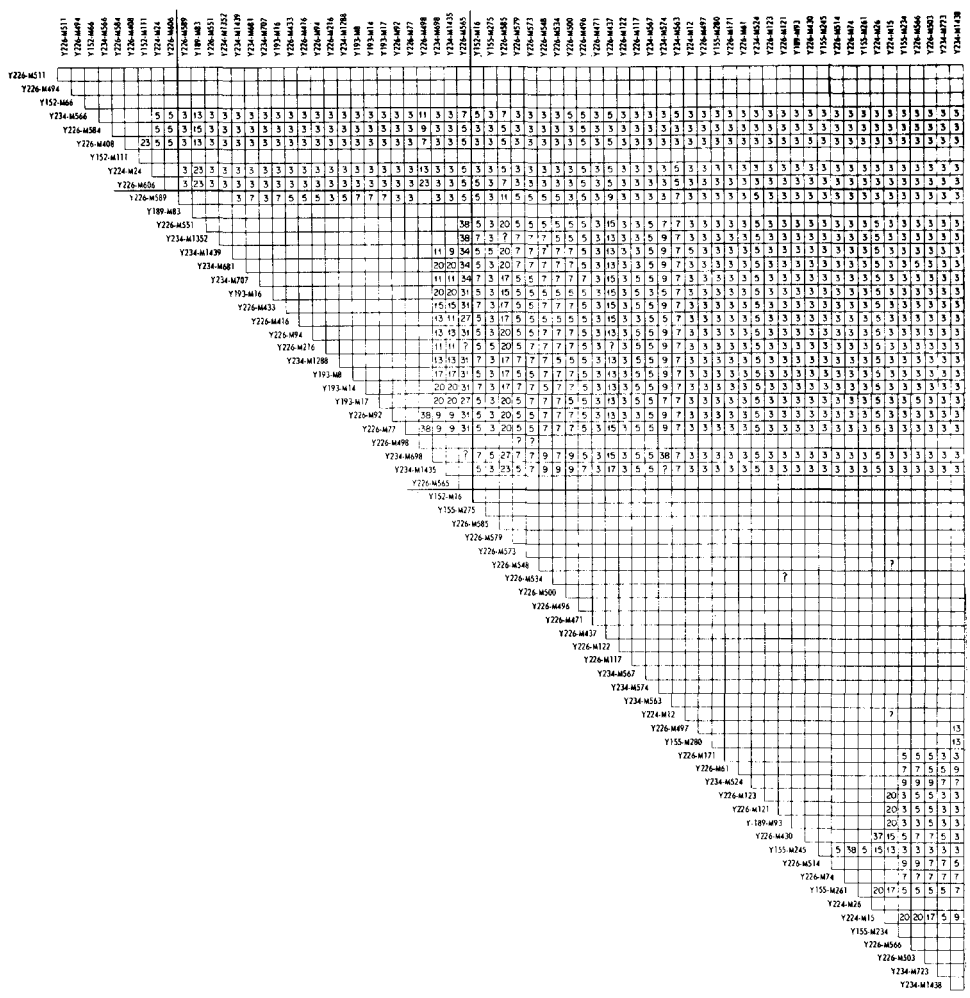
(giving an initial reading of about 0.600), and the concentration of other reagents was decreased to about one third of that used by AMES *et al.* (1960). Also, a potassium phosphate buffer was substituted for triethanolamine in preparing the Neurospora extracts. In preliminary experiments the triethanolamine buffer interfered with protein determination and the use of phosphate buffer was considered preferable.

RESULTS AND DISCUSSION

hist-3 *complementation map:* The data from the study of complementation are presented in Table 1. The 68 mutants used were in the proper genetic background

TABLE 1

Heterokaryon responses of 68 hist-3 *mutants in all possible combinations of pairs*



Mutant isolate numbers are at top and sides of table. Numbers in body of table indicate days between inoculation and positive response. Blanks mean no complementation in 45 days. Question mark indicates test result concealed by contamination.

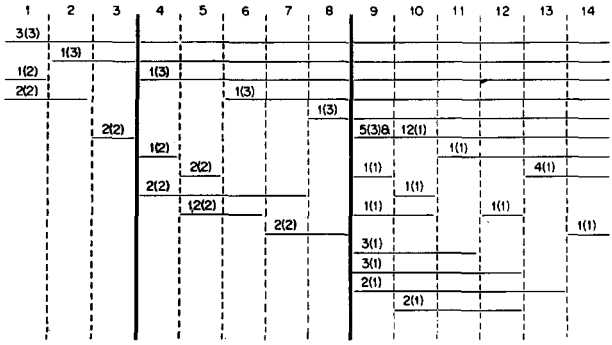


FIGURE 1.—Heterokaryon complementation map of 68 selected *hist-3* mutants. Accompanying each line is the number of mutants exhibiting the complementation characteristics represented by that line; the subgroup classification of the mutants is in parentheses. Complon (complementation unit) numbers are at top of figure.

for complementation (as shown by 3-day positive responses between two presumably nonallelic histidine-requiring mutants and these 68 mutants). Most of these also exhibited positive responses with other *hist-3* mutants in preliminary studies and were selected on this basis.

The complementation map derived from available data is presented in Figure 1. It is divided into 14 complons, which are numbered at the top of the figure. In the complementation map there is obvious polarity, with 27 complementing mutants starting at various positions on the left and extending through complon 14. Only three complementing mutants extend through complon 1. The latter mutants might be interpreted as exhibiting polarity in the reverse direction if they were not clustered together in the same region of the map.

Correlation between complementation map position and biochemical deficiencies: Information about these *hist-3* mutants is listed in Table 2 in a manner which permits one to observe how enzyme activity deficiencies and complon coverage are related to one another. Included are the results of histidinol dehydrogenase assays and of Pauly tests for histidinol accumulation in growth medium which provide the basis for the given subgroup classification. The subgroup classification is also indicated in Figure 1 along with each of the lines indicating complon coverage. The generalization may be made that subgroup 1 mutants cover one or more of the complementation units (complons) 9 through 14, subgroup 2 mutants are restricted to one or more of complons 1 through 8, and subgroup 3 mutants cover complons 9 through 14 and extend to the left of complon 9. Complons 9 through 14 apparently represent separate functions concerned with histidinol dehydrogenase, while complons 1 through 8 are related to the enzyme activities early in histidine biosynthesis. The apparent discrepancy that the complon 9 through 14 class of mutants includes five subgroup 3 mutants and 12 subgroup 1 mutants may be explained by the existence of an additional complon between complons 8 and 9 which cannot be detected by the mutants used here, but which is covered by subgroup 3 mutants and not by the subgroup 1 mutants. In fact, it can be seen in Table 1 that 4/5 of these subgroup 3 mutants show later complementation responses than do these 12 subgroup 1 mutants.

TABLE 2

Complementation map position and biochemical characteristics of some hist-3 *mutants*

Mutant number	Complons (complementation units) covered	Histidinol in medium (PAULY tests)	Histidinol dehydrogenase assay (μmole/min/mg protein)	Biochemical subgroup
Y226–M511	1–14*	0	0	3
Y226–M494	1–14*	0	0	3
Y152–M66	1–14*	0	.	.
Y234–M566	1–2	0	10.0; 10.4	2
Y226–M584	1–2	0	12.8	2
Y226–M408	1	0	13.7; 15.4	2
Y152–M111	2–14	0	0	3
Y224–M24	3	0	9.6; 8.4	2
Y226–M606	3	0	7.5	2
Y226–M589	4	0	1.2; 1.6	2
Y189–M83	4–14	0	0	3
Y226–M551	4–7	0	3.7; 4.6	2
Y224–M1352	4–7	0	3.0; 3.7	2
Y234–M1439	5–6	0	5.7; 5.8	2
Y234–M681	5–6	0	4.9; 5.1	2
Y234–M707	5–6	0	6.2	2
Y193–M16	5–6	0	7.9; 8.2	2
Y226–M433	5–6	0	10.4	2
Y226–M416	5–6	0	7.1	2
Y226–M94	5–6	0	7.1; 7.7	2
Y226–M216	5–6	0	8.3; 9.2	2
Y234–M1288	5–6	0	8.5; 9.0	2
Y193–M8	5–6	0	8.0; 9.5	2
Y193–M14	5–6	0	7.3; 7.7	2
Y193–M17	5–6	0	10.4	2
Y226–M92	5	0	8.2; 11.0	2
Y226–M77	5	0	8.2; 9.2	2
Y226–M498	6–14	0	0	3
Y234–M698	7–8	0	3.2; 3.3	2
Y234–M1435	7–8	0	3.7; 4.0	2
Y226–M565	8–14	0	0	3
Y152–M16	9–14	±	0	3 or 1
Y155–M275	9–14	+	.	1
Y226–M585	9–14	0	0	3
Y226–M579	9–14	±	0	3 or 1
Y226–M573	9–14	+	.	1
Y226–M548	9–14	+	.	1
Y226–M534	9–14	+	0	1
Y226–M496	9–14	0	0	3
Y226–M500	9–14	+	0	1
Y226–M471	9–14	+	0	1
Y226–M437	9–14	0	0	3
Y226–M122	9–14	+	.	1
Y226–M117	9–14	+	0	1
Y234–M567	9–14	+	.	1
Y234–M574	9–14	0	0	3
Y234–M563	9–14	+	0	1
Y224–M12	9–14	+	.	1

TABLE 2—Continued

Complementation map position and biochemical characteristics of some hist-3 *mutants*

Mutant number	Complons (complementation units) covered	Histidinol in medium (PAULY tests)	Histidinol dehydrogenase assay (μmole/min/mg protein)	Biochemical subgroup
Y226–M497	9–13	+	.	1
Y155–M280	9–13	+	.	1
Y226–M171	9–12	+	0	1
Y226–M61	9–12	+	.	1
Y234–M524	9–12	+	.	1
Y226–M123	9–11	+	.	1
Y226–M121	9–11	+	0	1
Y189–M93	9–11	+	.	1
Y226–M430	9–10	+	.	1
Y155–M245	9	+	.	1
Y226–M514	10–12	+	0	1
Y226–M74	10–12	+	.	1
Y155–M261	10	+	.	1
Y224–M26	11–14	+	.	1
Y224–M15	12	+	.	1
Y155–M234	13–14	+	.	1
Y226–M566	13–14	+	.	1
Y226–M503	13–14	+	0	1
Y234–M723	13–14	+	.	1
Y234–M1438	14	+	.	1
1710	too leaky	+	0	1
74A (wild type)		0	4.0; 4.7	wild type

* Noncomplementing mutants.

Relationship between complementation units covered and histidinol dehydrogenase activity: In Figure 2, the rightmost complon covered by a mutant is plotted against specific activity for histidinol dehydrogenase. The activity decreases as complon 9 is approached. Wild-type Neurospora (74A) has a specific activity of 4.4, which is exceeded by all but five of the mutants tested. The most likely interpretation is that a general enhancement of histidinol dehydrogenase activity results from a lack of end-product repression due to the block in histidine biosynthesis, and that superimposed upon this is an inhibitory effect which increases in intensity with decreasing distance from the histidinol dehydrogenase portion of the map.

Distinction between polarized and nonpolarized type of complementation pattern: Mutants have been classified as covering one or more of the complons related to histidinol dehydrogenase activity (9 through 14) or the complons related to reactions early in histidine biosynthesis (complons 1 through 8) or both. A different type of classification would distinguish between polarity mutants, which extend from some complon continuously through complon 14, and mutants with more restricted complon coverage, whose patterns are nonpolarized.

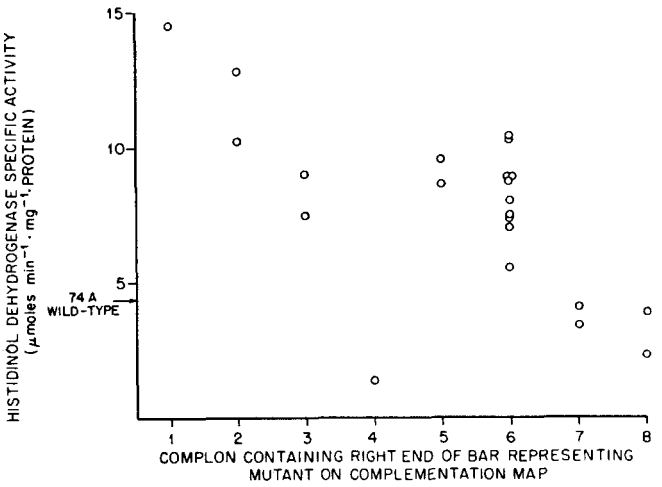


FIGURE 2.—Relation between distance of nonpolarized mutant from right end of complementation map and histidinol dehydrogenase specific activity of that mutant. (The complon-4 mutant Y226-M589 which exhibits activity of 1.4 here may be considered a double mutant, as it gives a negative heterokaryon test with the complon 6–14 mutant, Y226-M498.)

A correlation between mutagenic origin and type of complementation pattern of *ad-3B* mutants of Neurospora (DE SERRES 1964) suggests that there is a correlation between the type of genetic alteration and the type of complementation pattern. These studies indicate that complementing mutants exhibit one or two basic types of complementation pattern, either polarized or nonpolarized. They also suggest that in Neurospora nonpolarized patterns result from base-pair substitutions, while polarized patterns and noncomplementing mutants result from base-pair insertions and deletions. It is of interest in this regard that *hist-3* mutants with polarized patterns are frequently slower in their positive responses than mutants with nonpolarized patterns.

Relationship between proximity of pairs of mutants on complementation map and promptness of heterokaryotic growth response: WOODWARD, PARTRIDGE and GILES (1958) observed a correlation between distance separating pairs of *ad-4* mutants on a complementation map and promptness of heterokaryotic growth response. Many adjacent pairs of *hist-3* mutants grow in 3 days and so it seems more reasonable to point out a tendency for *hist-3* mutants (except the polarity mutants, which typically respond slowly) to fall into classes such that intraclass responses are slower than interclass responses. One such class includes mutants which cover complons 1, 2, and/or 3. These exhibit 5-day responses in combination with other restricted mutants within the class, while heterokaryotic growth occurs in 3 days if any of them is incubated with mutants covering only other complons. The other such classes include mutants which are restricted to complons 4, 5, 6, 7, and/or 8 and mutants (subgroup 1) which cover one or more of complons 9 through 14. It is interesting that the biochemical classification of complons is reflected in the behavior of mutants in complementation tests. Com-

TABLE 3

Characteristics of testers used for classifying randomly selected hist-3 *mutants by heterokaryon tests*

Tester number	Isolate number	Extent on previous complementation map (complons covered)	Biochemical subgroup
1	Y226–M408	1	2
2	Y226–M606	3	2
3	Y226–M589	4	2
4	Y226–M216	5–6	2
5	Y226–M430	9–10	1
6	Y226–M123	9–11	1
7	Y226–M61	9–12	1
8	Y155–M234	13–14	1

plons 1 through 3 are related to one early biosynthetic reaction, complons 4 through 8 are related to a different early reaction (A. AHMED, unpublished), and complons 9 through 14 are concerned with the terminal step in histidine biosynthesis. This would lead one to favor the prediction that three different enzyme molecules are associated with the three reactions.

Studies of complon coverage of randomly selected hist-3 *mutants:* 238 *hist-3* mutants induced by UV in 74A genetic background were selected at random to provide an estimate of relative frequency of mutants with different complementation patterns. Eight *hist-3* mutants scattered across the complementation map were used as testers and are listed and described in Table 3. Concentrations of conidia were adjusted (by visual comparison with a standard subjected to haemocytometer counts) to about 5×10^5 conidia of tester and 5×10^5 or more of the mutant to be tested in 2 ml of Fries' minimal medium per tube. Tubes were incubated and observed for only 10 days.

The testers with which each of the 238 mutants produced negative results were noted. These results are presented in Table 4. A negative test indicates that the mutant being tested covers at least one complon which the tester covers; therefore, a minimal complon coverage can be ascribed to each mutant. We have observed that complons 9 through 14 are concerned with histidinol dehydrogenase activity and complons 1 through 8 with early biochemical functions in histidine biosynthesis. It is possible to use this and the minimal complon coverage of a mutant to infer a probable biochemical subgroup classification, which is also indicated in Table 4. These same 238 mutants were also subjected to tests for histidinol accumulation, which distinguishes subgroup 1 mutants (accumulators of histidinol) from the other two subgroups. In 235 cases the predictions based on complementation studies were actually confirmed by the accumulation tests.

These studies with the randomly selected mutants confirm the sequence of complons established in the map in Figure 1 and support the generalization that complons 9 through 14 are concerned with histidinol dehydrogenase and complons 1 through 8 with reactions early in histidine biosynthesis. They also provide the information that subgroup 3 mutants are most frequent (with completely

TABLE 4

Results of heterokaryon tests of 238 randomly selected ultraviolet-induced mutants with testers described in Table 3

Testes which failed to complement	Minimal complon coverage	Distribution of the random *hist-3* mutants	
		Number	Percent
Subgroup 3—no histidinol dehydrogenase activity; no histidinol accumulation			
1–8	1–13 (non-complementary)	109	46
2–8	3–13	2	1
3–8	4–13	23	10
4–8	6–13	12	5
Subgroup 2—histidinol dehydrogenase activity; no histidinol accumulation			
1	1	1	0.4
3–4	4–5	2	1
4	5 or 6	8	3
4–5*	6–9	1	0.4
Subgroup 1—no histidinol dehydrogenase activity; histidinol accumulation			
5–7	9 or 10	19	8
5–8	10–13	45	19
8	13 or 14	16	7

* This result suggests that tester 5 extends farther to the left than testers 6 and 7 and indicates the tentative existence of an additional complon between the present complons 8 and 9.

noncomplementing mutants being the largest single class) and that subgroup 2 mutants are the least frequent in this sample of UV-induced mutants. The high incidence of mutants with polarized complementation patterns is also clear in these data, for 98 of the complementing mutants fail to complement with the complon 13–14 tester, while only one of the complementing mutants fails to complement with the complon 1 tester.

The analysis of *hist-3* mutants may be contrasted with the analysis of *ad-3* mutants (DE SERRES 1956) where two separate loci (*ad-3A* and *ad-3B*) have been defined on the basis of heterokaryon tests and mutant intercrosses. The findings that more than 60 percent (147/238) of a series of randomly selected *hist-3* mutants span the boundary separating complons 8 and 9 and that 46 percent (109/238) are completely noncomplementing indicate that the *hist-3* mutants share a close functional relationship. The *hist-3* region could be presumed to constitute a single uninterrupted piece of genetic material which is read continuously, while the two loci in the *ad-3* region are read separately.

It is striking that a small genetic region (originally interpreted on the basis of genetic data as a single locus) should be concerned with the production of three different enzymic activities in such a way that these activities may be eliminated separately or, more often, jointly by mutational events localized at particular sites within that region. Crossing experiments designed to elucidate the genetic basis for this biochemical diversity among *hist-3* mutants are reported separately (WEBBER 1965).

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SUMMARY

Sixty-eight *hist-3* mutants were tested in all possible combinations of pairs in prolonged heterokaryon tests for interallelic complementation. The interaction matrix defines a complementation map consisting of 14 complementation units (complons), with all mutants represented as continuous straight lines.

Tests for histidinol accumulation and for *in vitro* histidinol dehydrogenase activity show the following correlations: (a) Subgroup 1 mutants (deficient for histidinol dehydrogenase) are all located in complons 9 through 14. (b) Subgroup 2 mutants (deficient for one or two early reactions in histidine biosynthesis) are located in complons 1 through 8. (c) Subgroup 3 mutants (deficient for histidinol dehydrogenase as well as one or two early reactions) cover at least complons 9 through 14 and typically extend into the other half of the map.

Mutants with nonpolarized complementation patterns can be separated into three classes on the basis of the type of heterokaryon response, with tests within classes being slower than interclass tests. One line of demarcation falls between complons 8 and 9 (separating subgroup 1 mutants from subgroup 2 mutants); the other falls between complons 3 and 4. These three classes of mutants have different biochemical deficiencies.

Randomly selected ultraviolet-induced *hist-3* mutants were mapped roughly by heterokaryon tests with eight tester strains. Sixty-one percent of these extend into both halves of the map while 46 percent probably are noncomplementary with other *hist-3* mutants, suggesting a close physiological relationship among various types of *hist-3* mutants.

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