COMPLEMENTATION STUDIES WITH ISOLEUCINE-VALINE MUTANTS OF NEUROSPORA CRASSA[1]

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IN recent years a number of investigators have presented the results of experiments concerned with complementation between mutants defective for a single genetic character. These results and their probable significance have been reviewed and discussed by BEADLE (1960), CASE and GILES (1960), FINCHAM (1960), and YANOFSKY and ST. LAWRENCE (1960).

The present work includes an intragenic complementation study at each of two loci concerned with isoleucine-valine biosynthesis. It was hoped that certain features of the complementation interaction could be clarified by a rather extensive program of testing. Specifically, understanding of the following problems was sought. Are the subgroups, of which the complementation map is composed, discrete? Does each genetic locus have associated with it a unique and characteristic pattern of such subgroups? Would linearity of the complementation map be observed in these systems?

MATERIALS AND METHODS

Induction and selection: Mutants were obtained by the "inositol-less death" selection procedure described by LESTER and GROSS (1959). The strain used in all induction experiments was *inos* 89601a which originated in a sample of nitrogen mustard treated spores descended from the standard wild cross Em 5256A × Em 5297a. Conidia were harvested from cultures grown at 35° on 20 ml of the complete medium described by HOROWITZ (1947).

The mutagens used, conditions of treatment and appropriate references are given in Table 1. The conidial concentrations at the time of treatment, as determined by optical density and comparison to a standard curve, were in the range $3–10 \times 10^7$ conidia per ml. All chemical treatments were terminated by dilution into cold M/15 phosphate buffer of *p*H 7.0. In all experiments, except 1, 2 and 3, the shaking step recommended by LESTER and GROSS (1959) was included.

All isoleucine-valine mutants were obtained on plates overlayed (after 3–4 days selection) with 5 ml of a minimal medium described by VOGEL (1956) to which was added agar 6.5 mg/ml, glucose 2.0 mg/ml, glycerol 2.0 mg/ml, sorbose

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TABLE 1

Mutagenic treatments

Mutagen	Abbrev.	Conc. or dose	Duration of treatment	Ml of conidial suspension treated	Measured conidial survival	References
Ultraviolet light*	UV	....	50 sec	10	30%	
X-irradiation†	X-ray	2500r/min	40 min	20	20%	
Nitrogen mustard (methyl-bis [beta-chloroethyl]-amine hydrochloride)	NM	0.0025 M	30 min	20	..	KÖLMARK and WESTERGAARD 1953
5-bromodeoxyuridine‡	5-BDU	....	..	..	..	
Tert-butyl-hydroperoxide	TBHP	0.09 M	30 min	20	..	DICKEY, CLELAND and LOTZ 1949
Ethyleneimine	ETIM	0.05 M	40 min	20	..	KÖLMARK and WESTERGAARD 1953
β-propiolactone	β-PL	0.004 M	30 min	20	..	SMITH and SRB 1951
Hydrogen peroxide with formaldehyde	HP+F	0.06 M H_2O_2 0.03 M HCHO	30 min	20	..	KÖLMARK and WESTERGAARD 1953
Nitrous acid§	HNO_2	0.005 M	96 min	11.6	..	KÖLMARK and DESERRES 1960

* The dose was delivered by a 30 Watt General Electric Germicidal Lamp to a suspension in an open petri dish (radius 4.5 cm) 9 cm from the lamp.
† The dose was delivered by a 250 kv Westinghouse Industrial X-ray Unit with no filtration to a suspension in an open petri dish 36 cm distant from the window.
‡ Conidia were harvested from cultures grown on 20 ml of HOROWITZ complete medium (HOROWITZ 1947) supplemented with 200μg/ml 5-BDU.
§ To initiate treatment four mg of $NaNO_2$ were added to the conidial suspension in 0.1 M acetate buffer (pH 4.6).

15 mg/ml, inositol 1 mg/ml, L-isoleucine 200 μg/ml and L-valine 200 μg/ml. Valine mutants were obtained from plates receiving an overlay similar to the above except that isoleucine and valine were replaced by 3.0 mg/ml casamino acids and the inositol concentration was 35 μg/ml.

Complementation tests: Complementation tests were regularly performed by a modification of the method of CATCHESIDE and OVERTON (1958). Large petri dishes (radius 7 cm) were used which contained 50 ml of Vogel's medium supplemented with sorbose 5.0 mg/ml, sucrose 1.0 mg/ml, inositol 10 μg/ml and agar 20 mg/ml. Conidial suspensions were prepared from cultures freshly grown on complete medium. Although conidial concentrations were not usually measured, complementation results were found to be regularly reproducible as long as the suspensions were visibly turbid (i.e., above 5×10^6 conidia/ml). Tests carried out with more dilute suspensions were found to be unreliable. Inocula were spotted on the plates with a wire loop, each spot receiving conidia from two mutants. As many as 45 spots could be placed on individual plates. These were stored at 25° and growth was recorded daily. Overgrowth of spots by neighboring, early-positive tests usually limited the usefulness of plates to from six to ten days.

Another, more sensitive, method was also employed for complementation testing. Small test tubes (75 mm by 10 mm), each containing 1 ml of Vogel's minimal medium supplemented with inositol 10 μg/ml, sucrose 10 mg/ml, glycerol 10 mg/ml, L-valine 100 μg/ml, L-isoleucine 100 μg/ml and agar 20 mg/ml, were prepared. To each tube inocula from two mutants were added. After three days growth at 25°, conidia were transferred to corresponding tubes containing media identical to the above except for the omission of isoleucine and valine. Observations of complementation could then be recorded during a 20 day period. However, because of the considerable extra work involved for each test, the use of this method was limited to checking the results obtained by the plate method.

Crosses: Crosses were made on Westergaard medium (WESTERGAARD and MITCHELL 1947) which contained L-isoleucine 100 μg/ml, L-valine 100 μg/ml, inositol 10 μg/ml, and several other amino acids. Recombination frequencies were obtained by plating out appropriate dilutions of random spores on both isoleucine-valine supplemented, and minimal media. After 30 minutes of heating at 60°, the plates were placed at 35° for about 24 hours. Counts were made with a low power binocular microscope when the colonies were still quite small. Also determined was the percent of spores not germinating on plates supplemented with isoleucine and valine.

RESULTS

Mutant yields: In all, 612 mutants, allocated to 23 series (Table 2), were obtained. All mutants were capable of growth on medium supplemented with isoleucine and valine, but not on minimal medium. Each mutant was designated by two numbers, the first indicating the series in which it was obtained and the second the order in which it was found in that series. Thus, mutant 18–13 was the 13th mutant found in series 18. The mutants of Experiment I were unique in that they were obtained from plates overlayed with minimal medium contain-

TABLE 2
Yields and distribution of mutants

Exp.	Series	Mutagen	Colonies picked	Number of mutants	Percent yield	Distribution				
						val-1	*val-2*	*iv-1*	*iv-2*	*iv-3*
I	1	UV	49	8	16.3	4	4	0	0	0
II	2	UV	561	20	3.6	0	0	0	1	19
III	3	UV	97	2	2.1	0	0	0	0	2
IV	4	UV	450	262	58.2	0	0	0	4	258
V	5	UV	53	23	43.4	0	0	0	1	22
VI	6	UV	101	56	55.5	0	0	0	0	56
VII	7	X-ray	18	1	5.6	0	0	0	1	0
	8	NM	246	3	1.2	0	0	0	1	2
	9	HP+F	400	0	0	0	0	0	0	0
	10	UV	400	15	3.8	0	0	1	4	10
	11	spontaneous	400	1	.25	0	0	0	0	1
	12	5-BDU	408	1	.25	0	0	0	1	0
VIII	13	UV	400	18	4.5	0	0	12	3	3
	14	X-ray	100	3	3.0	0	0	1	2	0
	15	NM	296	49	18.2	0	0	40	5	4
	16	TBHP	95	4	4.2	0	0	0	0	4
	17	ETIM	180	14	7.8	0	0	12	0	2
	18	β-PL	273	13	4.8	0	0	4	0	9
	19	HP+F	400	23	6.0	0	0	5	9	9
IX	20	UV	75	27	36.0	0	0	0	17	10
	21	NM	106	9	8.5	0	0	1	3	5
	22	HNO_2	30	2	6.7	0	0	1	0	1
	23	HP+F	465	58	12.5	0	0	1	42	15
Totals				612		4	4	78	94	432

ing casein hydrolysate, while in all other experiments an isoleucine-valine supplement was used.

Factors such as age of harvested conidia, conidial concentration at time of mutagen treatment, conidial concentration at time of plating and duration of selection are all expected to affect the yield of mutants (LESTER and GROSS 1959; KÖLMARK and WESTERGAARD 1953). Each of the experiments was known to vary with respect to these factors, and this, at least partially, accounts for the significant differences in distribution of mutant types from experiment to experiment (Tables 2 and 4). For each of the series within Experiments VII, VIII, and IX, these factors were held very nearly constant, from one series to the next, and only the mutagens used were changed.

Grouping of mutants by complementation: On the basis of complementation tests all isoleucine-valine mutants were placed in three groups, and the valine mutants into two additional groups. These have been designated[3] *iv-1*, *iv-2*, *iv-3*,

[3] The designation *iv-1* was first used by BARRATT, NEWMEYER, PERKINS and GARNJOBST (1954) as an assignment for mutant 16117 and its possible alleles. The designation *iv-2* was also introduced by these authors for alleles of mutant 39709, which on the basis of unpublished data was closely linked to 16117. The use of *iv-1* in the present work is consistent with this original usage. However 39709 was found to be heterokaryon incompatible with the mutants used in this study and there is no additional mention of it in the literature. Therefore it is not certain that present and original usage are consistent.

val-1 and *val-2*. All tests involving two mutants from different groups were always positive within a day after inoculation. The data from all intergroup tests are summarized in Table 3. The distribution of mutants, obtained in each series, among the various groups, is given in Table 2.

No complementation was observed between members of either the *val-1* or *val-2* group. Mutants in the *iv-1* group were all observed to grow on minimal medium between four and six days. For the other groups, the proportion of mutants displaying growth on minimal medium before five days was not more than a few percent of the total number. Among the 1,012 *iv-1* × *iv-1* pairwise complementation tests that were performed, several were positive 2–3 days before either mutant alone revealed growth. However, because of the complication of slow growth on minimal medium, this study was not pursued further.

The complementation behavior of isoleucine-valine mutant 16117, obtained by BEADLE and TATUM (1945), clearly placed it in the *iv-1* group. This mutant, in addition, displayed the slow growth on minimal medium characteristic of all *iv-1* mutants. It has been shown (ADELBERG and CAUGHLIN 1955) that 16117 accumulates the intermediates α,β-dihydroxyisovaleric acid and α,β-dihydroxy-β-methylvaleric acid (Figure 1), and has greatly diminished dehydrase activity with respect to these compounds (MEYERS and ADELBERG 1954). It is inferred from this evidence, and the recombination data to be presented, that *iv-1* mutants are defective in the dehydrase step of isoleucine-valine biosynthesis.

Complementation tests carried out on minimal medium supplemented with either L-valine alone, or L-isoleucine alone, gave results indistinguishable from tests carried out in the usual manner on unsupplemented minimal medium.

The iv-2 *group:* The 94 mutants allocated to the *iv-2* group were tested in 832 pairwise combinations. Eighteen of these mutants were found to complement with at least one other in the group. All possible pairwise tests among these 18 were performed. On the basis of the results a completely self-consistent linear complementation map was formulated (Figure 2). The mapping procedure is as follows: Results of pairwise growth tests are scored in the form of a matrix of positive and negative responses. Mutants are found which do not complement

TABLE 3

Intergroup complementation tests

Groups	Number of tests performed (all positive)
iv-1 + *iv-2*	620
iv-1 + *iv-3*	355
iv-1 + *val-1*	77
iv-1 + *val-2*	78
iv-2 + *iv-3*	1445
iv-2 + *val-1*	35
iv-2 + *val-2*	39
iv-3 + *val-1*	144
iv-3 + *val-2*	152
val-1 + *val-2*	16

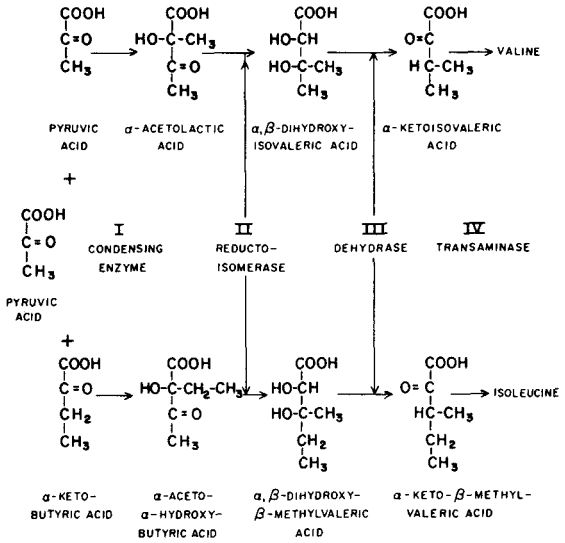

FIGURE 1.—Current concept of metabolic pathways for isoleucine and valine biosynthesis. (Based on WAGNER, RADHAKRISHNAN and SNELL 1958 and RADHAKRISHNAN, WAGNER and SNELL 1960).

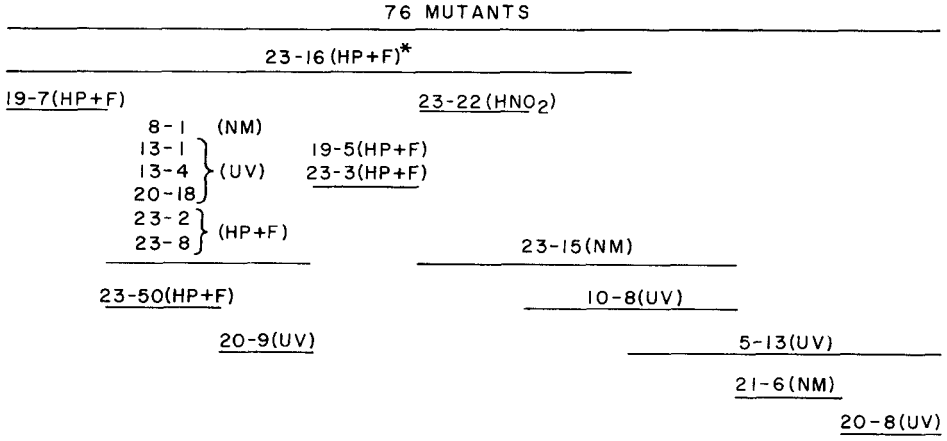

FIGURE 2.—Complementation map of the *iv-2* group. The individual mutants belonging to each subgroup, along with their mutagenic origins, are listed. The abbreviations used are: ultraviolet light, UV; nitrogen mustard, NM; hydrogen peroxide with formaldehyde, HP+F; and nitrous acid, HNO_2. The mutagenic origins of the "complete overlap" mutants are given in the text. *Mutant 23–16 complemented after three days with 5–13, but only after seven and six days with 21–6 and 20–8 respectively.

with two or more mutants which, in turn, do exhibit complementation. These noncomplementing or "overlap" mutants provide a basis for the construction of an ordered map. All mutants having the same pattern of complementation are placed in one subgroup represented on the map by a line. Noncomplementing

subgroups are represented by overlapping lines, whereas lines representing complementing subgroups are not overlapped. If the data allows every subgroup to be designated by a single uninterrupted line, the complementation map is said to be linear. However, the positions of all the subgroups were not uniquely determined, and therefore more than one linear map could have been derived from the data. For instance, the positions of 21–6 and 20–8 could have been reversed. Almost all positive responses were initiated between three and five days after inoculation. There was some tendency for heterokaryotic growth of a mutant combination to be more vigorous as the apparent number of subgroups separating mutants on the map increased. However, one case of exceptional behavior was clearly established in repeated tests. Mutant 23–16 initiated a positive response with 5–13 three days after inoculation, whereas with the more "distant" 21–6 and 20–8, complementation was not observed until seven and six days, respectively.

The 76 completely overlapping mutants had the following mutagenic origins: 42, hydrogen peroxide with formaldehyde; 23, ultraviolet light; seven, nitrogen mustard; three, X-rays; and one, bromodeoxyuridine. Of particular interest were six mutants, representing three different mutagenic origins, which displayed identical complementation patterns (Figure 2 second subgroup from left). This result was characteristic, to a greater extent, of mutants in the *iv-3* group, and therefore its possible significance will be discussed in the following sections.

The iv-3 *group*: On the basis of a program of testing in which the 432 *iv-3* mutants were subjected to about 10,500 pairwise complementation tests, the map shown in Figure 3, was formulated. The complementation behavior of 428 of the mutants was completely consistent with this linear representation, but the remaining four could not be reconciled with it. Three of these would not conform to any linear representation. The properties of these mutants are discussed below.

The most interesting feature of the results was that the complementation map contained only a few subgroups, i.e. among 428 mutants there existed only six patterns of complementation. However, 325 of these were C mutants derived from UV treatment. This representation with only six subgroups was initially

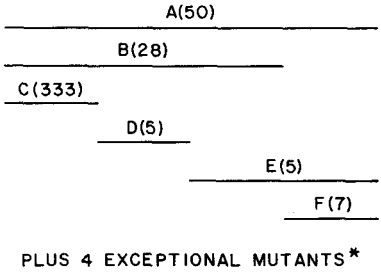

FIGURE 3.—Complementation map of the *iv-3* group. Each subgroup is given a letter designation after which is the number of mutants found in that subgroup. *Four *iv-3* mutants displayed patterns of complementation incompatible with this representation. Three of these could not be reconciled with any map in which all the subgroups are represented by uninterrupted line segments. For further details, see text.

observed early in the testing program when only UV induced mutants were available. Of the 52 non-UV mutants subsequently obtained (Table 4) 25 did not complement and were placed in subgroup A, 23 could be placed in one of the other five established subgroups, and four gave exceptional results.

The number of possible pairwise tests (T), where n is the number of mutants tested, is $\frac{1}{2}\, n(n-1)$. When n is 432, T is 93,096. The number of tests actually performed, 10,500, was only 11 percent of this total. Therefore, it would seem advisable to mention some of the factors involved in choosing those tests which were carried out. Initially mutants 4–1 to 4–65 were tested in all pairwise combinations, and then arranged in classes according to their pattern of complementation. Mutants representing each class were next used as testers to classify systematically the other mutants. Any newly tested mutant not conforming to the established pattern of complementation, was, after rechecking, itself used as a tester. Classifications thus arrived at were subjected to numerous cross-checks. Although only 3,000 of the 55,278 possible C + C tests were actually performed (all giving negative results), it seemed unnecessary to prolong this phase of the testing program. The omitted C + C tests account for more than half the total tests not carried out.

TABLE 4

Distribution of iv-3 *mutants found in successive selection experiments**

Series no.	Mutagen	A	B	C	D	E	F	Exceptions	Totals
1	UV	0	0	0	0	0	0	0	0
2	UV	3	5	1	5	0	5	0	19
3	UV	0	1	1	0	0	0	0	2
4	UV	7	4	246	0	1	0	0	258
5	UV	1	1	20	0	0	0	0	22
6	UV	2	2	50	0	2	0	0	56
7	X-ray	0	0	0	0	0	0	0	0
8	NM	1	1	0	0	0	0	0	2
9	HP+F	0	0	0	0	0	0	0	0
10	UV	2	3	4	0	0	1	0	10
11	Spont	0	1	0	0	0	0	0	1
12	5-BDU	0	0	0	0	0	0	0	0
13	UV	3	0	0	0	0	0	0	3
14	X-ray	0	0	0	0	0	0	0	0
15	NM	1	2	0	0	0	0	1	4
16	TBHP	3	0	1	0	0	0	0	4
17	ETIM	0	1	0	0	0	1	0	2
18	β-PL	6	0	1	0	0	0	2	9
19	HP+F	3	2	3	0	1	0	0	9
20	UV	7	0	3	0	0	0	0	10
21	NM	2	1	2	0	0	0	0	5
22	HNO_2	0	0	1	0	0	0	0	1
23	HP+F	9	4	0	0	0	1	1	15
	Total	50	28	333	5	4	8	4	432
	UV	25	16	325	5	3	6	0	
	non-UV	25	12	8	0	1	2	4	

* Several mutants, which grew on minimal medium to the extent that classification was interfered with, were not included.

There tended to be a correlation between the vigor of heterokaryotic growth and the pair of subgroups represented in the heterokaryon. Thus the complementation interactions between mutants in the F and C groups were characterized by abundant growth starting on the first day after inoculation. The same was true of E + C and F + D, whereas E + D heterokaryons took about two days to initiate growth. B + F positive tests were variable in time of initiation but occurred, on the average, about five days after inoculation. Growth in these cases was always scanty. The tube method for detecting complementation (see Materials and Methods) was used mainly for checking the B + F combinations, and also D + C tests which grew after an average of 11 days, with even more variability, and gave exceedingly scanty growth response.

Two of the exceptional mutants, 8–10 and 23–56, did not complement with each other and were in all respects very similar. Neither complemented with A, E or F mutants. Both complemented readily with all B mutants tested. 18–10 gave a positive response with 24 B mutants after an average of about two days, whereas 23–56, in tests with 22 B mutants took about four days. Both complemented slowly with 60 percent of the C mutants (an average of five days in each case) and would not complement at all with 40 percent of the C's. To represent these mutants on the complementation map would inevitably require two nonadjacent line segments as well as a subdivision of group C. The new subgroups arising because of the exceptional mutants certainly have an equal claim to letter designations. However, in order to make these assignments with any surety, it would have first been necessary to have established the complete complementation spectrum of all the exceptional mutants. To accomplish this, with the required resolution, using the techniques now available, would have been a considerable undertaking.

The two remaining exceptional mutants (15–7 and 18–13) did complement with each other. 15–7 acted, in general, like a C mutant, although unlike C's, it complemented with about 40 percent of the B mutants after an average of eight days. Although there was insufficient data to completely characterize mutant 18–13, its behavior clearly did not fit the established pattern and was in addition incompatible with linearity.

Mutants representing the *iv-1*, *iv-2* and *iv-3* groups, in addition to *inos* 89601, have been assayed for the enzymatic ability to convert pyruvic acid to α-acetolactic acid essentially as described by RADHAKRISHNAN and SNELL (1960). A representative mutant in both the *iv-1* and *iv-2* groups, as well as *inos* 89601, displayed substantial activity for these reactions, whereas *iv-3* mutants had little if any activity. This preliminary evidence suggests that *iv-3* mutants are blocked in Step I (Figure 1) of the biosynthetic pathway.

An *iv* mutant, 7110, has been shown by WILSON and ADELBERG (1957) to accumulate several organic acids, the principal ones being citramalic and α,β-dimethylmalic acids. By complementation this mutant belonged to the *iv-3* group. The explanation for these accumulation products is unknown. Further work on the enzymology of Step I is now in progress.

Crosses: Preliminary crosses were performed in order to check to some extent

the groupings indicated by complementation tests. Mutant 16117A (*iv-1*), assigned to the right arm of linkage group V (BARRATT *et al.* 1954), was crossed to members of each of the five groups and proved to be unlinked or distantly linked to mutants and the *val-1*, *val-2* and *iv-3* groups. In the 12 fertile crosses between 16117 and various *iv-2* mutants, linkage was observed in every case. The values varied between 2.6 and 8.6 map units and had an average of 6.3. Spore germination in these crosses was between 40 and 90 percent. In the single fertile cross to another *iv-1* mutant (10–9), no prototrophs were observed among approximately 2,000 germinated random spores. In this case there was 21 percent germination. Therefore mutants 16117 and 10–9 represent defects at either identical or very closely linked sites. This evidence suggests that the three *iv* groups represent three genetic loci. Two of these (*iv-1* and *iv-2*) are in linkage group V, about 6.3 map units apart, and both are unlinked to *iv-3*.

DISCUSSION

The most apparent conclusion from these interallelic complementation studies is that all of the *iv-2* data and the large majority of the *iv-3* data are compatible with simple linear complementation maps. In addition, the data are in general accord with WOODWARD's observation (WOODWARD 1959) that the vigor of heterokaryotic growth between two mutants increases with "distance apart" on the complementation map. At the present stage of understanding it is difficult to know how to assess the importance of the four *iv-3* mutants whose complementation behavior differs from the established pattern, and especially the three of these which will not conform to a linear map.

On the basis of these results, the hypothesis may be formulated that *each locus has associated with it a unique and characteristic pattern of discrete complementation subgroups*, and that the observations in a complementation study reflect this pattern. It is, furthermore, assumed that this pattern reflects the molecular structure of the enzyme controlled by the locus.

The main support for this hypothesis is the result that the very large number of *iv*-3 mutants which were found, proved after extensive testing, to belong to a relatively small number of subgroups. Also of consequence was the finding that, of 18 complementing *iv-2* mutants, six fell into one subgroup. It is predicted that the number of *iv-2* subgroups will continue to remain small with the accumulation of more *iv-2* mutants.

The validity of the hypothesis depends, in part, on how safely grounded are the subgroup assignments. It can be asked, with what degree of certainty are the mutants, allocated to a single subgroup, identical to each other in complementation behavior? When successive mutants within a subgroup are tested against mutants in subgroups that are not adjacent on the map, very uniform positive results are obtained. However, when the tests involving "adjacent" subgroups are considered, qualifications do arise. These tests give the least vigorous heterokaryotic growth and are, in fact, often variable in expression. However, increased care and repetition tends to minimize variations. It therefore seems probable that

it is the insensitivity of the procedures generally used for detecting complementation, when applied to weakly positive tests, rather than a lack of subgroup discreteness that accounts for this variability.

A note of caution should be included concerning limitations of the heterokaryotic growth assay as a measure of the primary complementation interaction which undoubtedly occurs at or close to the molecular level. The possibility must be entertained that, if a more direct feature of complementation such as enzymatic activity, were regularly screened for, the observed pattern of interactions at a given locus would be different. Certainly with the greater resolution offered by enzyme assay the observed pattern could be modified.

Also it is easy to envision situations in which enzymes active *in vitro* would not be active *in vivo* and thus not support growth. For example experiments by SUSKIND and KUREK (1959) indicate that a potentially active form of tryptophan synthetase is inhibited by high levels of metal ion in the cytoplasm. If enzymes of this type were to form by complementation, the growth and enzymatic assays would yield conflicting results.

Another limitation of the growth assay arises because mutants which are capable of some growth on minimal medium are difficult to handle experimentally. The selective omission of this class of mutants from testing schedules may lead to a bias in the observed complementation patterns. In the present experiments a few mutants were left out of the tabulations because their slow growth on minimal medium interfered with the tests necessary for classification. However, from the tests that could be performed, there was no indication of unusual complementation behavior.

Ideally, a completely general test for complementation would be independent of large increases in enzymatic activity and measure interaction, *per se*. Present methodology precludes a consideration of the generality of interaction between products of allelic mutants coexisting in a heterokaryon. Although, the application of immunological or physiochemical tests seems impractical at this time, the study of enzymes produced in heterokaryons of wild type and mutant may give some indication of the generality of interaction.

Interpretations of the results are subject to further reservations imposed by a lack of knowledge concerning two factors which could certainly bias the distribution of mutant types. These are, mutational "hot spots" (BENZER and FREESE 1958) and selection (during the experiments by which the mutants were obtained). In phage T_4 it has been shown that the spectra of "hot spots" at a genetic locus, induced by unrelated mutagens, show little, if any, overlap (BRENNER, BENZER and BARNETT 1958). Therefore, the observation that 23 of 27 non-UV induced *iv-3* mutants gave the same few complementation patterns observed for UV induced mutants, argues against the possibility that similarity of behavior among mutants primarily reflects "hot spot" contributions.

No estimation can be made at present concerning the effect of selection on the distribution of mutants among the various subgroups.

WAGNER, SOMERS and BERGQUIST (1960) have recently reported the results of experiments also concerned with isoleucine-valine mutants of Neurospora.

They concluded that clustered in a single complementation group were three kinds of mutants. Those allocated to one end of the complementation map were deemed defective in the reductoisomerase step (Figure 1), and those at the other end, in the dehydrase step. Mutants located in the middle were postulated to be defective in both steps. Their recombination data were used to assign these mutants to a small region of the genetic map (about four map units) in the right arm of linkage group V. One mutant, 16117, also used in the present study, was placed at the dehydrase end of their complementation map.

Our results place 16117 in what we have designated complementation group *iv-1*. All pairwise tests of these mutants with those we have placed in group *iv-2* were clearly positive within one day, without any indication of ambiguity (Table 3). Recombination data, albeit limited, indicate that mutants in group *iv-2* are similar to those reported by WAGNER, *et al.* (1960) which lack only the reductoisomerase and which cluster at the end distal from 16117 in their complementation map.

Thus it appears that the present results differ from those of WAGNER, *et al.* (1960) in that no mutants were found which exhibited complementation behavior corresponding to that of mutants placed in the middle group described above. Whether this difference is attributable to differences in mutant selection or complementation testing techniques remains to be examined.

SUMMARY

A total of 616 mutants capable of growth on a medium supplemented with isoleucine and valine, but not on minimal medium, were obtained through the use of nine different mutagens. On the basis of heterokaryon complementation tests all of the mutants could be allocated to five groups. These groups were designated *val-1*, *val-2*, *iv-1*, *iv-2* and *iv-3*. Mutants in the *val-1* and *val-2* groups required valine as the sole supplement. Members of the *iv-1* group were probably blocked in the dehydrase step of isoleucine-valine biosynthesis, and were characterized by slow growth between four and six days after inoculation on minimal medium. An extensive program of complementation testing was performed among mutants within both the *iv-2* and *iv-3* groups. The results of these tests allowed, in each case, the formulation of a complementation map. However, three *iv-3* mutants were found which were not compatible with a linear pattern. An interesting feature of the *iv-3* studies, and to some extent of the *iv-2* studies, was the finding that among successive mutants the same patterns of complementation interaction often recurred. Mutants with the same complementation behavior constitute a complementation subgroup. Each of these subgroups usually contained mutants representative of several different mutagenic treatments. The hypothesis was considered that this observed clustering reflects an intrinsic property of the genetic locus—namely that each locus has associated with it a unique pattern of discrete complementation subgroups.

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