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# *ON THE MECHANISM OF COMPLEMENTATION AT THE LEU-2 LOCUS OF NEUROSPORA**

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Two different defective alleles of a gene determining the structure of a specific protein when in the *trans* configuration often complement each other yielding a protein with catalytic activity. This phenomenon has been referred to as inter-allelic complementation[1] and has been observed in diploids,[2] partial diploids,[3] and heterokaryons formed between haploid organisms.[4] Assuming only that mutant alleles which fail to complement each other share a defective segment in interacting structures while those pairs of alleles that do complement each other do not share overlapping defects, it has been possible to map a series of complementing alleles in a linear overlapping array.[1] The surprising feature of most complementation maps is that they can be represented topographically as a straight line. Recently, two exceptions to the usual "endedness" of such linear maps have been reported. Both the *dumpy* locus of *Drosophila*[2] and the rII A cistron of T4 bacteriophage[5] have been shown to be continuous.

In this report data are presented that indicate that the complementing *leu*-2 mutants of *Neurospora crassa* can be mapped in a linear continuous overlapping display. In addition, an interrelationship is demonstrated between complementation of *leu*-2 mutants and mutation at the related but unlinked locus, *leu*-3. Results are presented of an experiment expressly designed to test an hypothesis that relates complementation between *leu*-2 mutants to an interaction between polypeptides coded by the *leu*-2 and *leu*-3 genes in the formation of a polymer (at least a tetramer) with enzymatic activity.

*Methods and Materials.*—One hundred fifty-eight *leu*-2 mutants (alleles of *leu* 37501 which in turn, is assumed to be an allele of *leu* 8839[6]) were used in the complementation analysis. All were derived from the inositol requiring mutant 89601 by the inositol-less death enrichment procedure of Lester and Gross.[7] Ultraviolet light was the mutagen employed in obtaining all mutants except D268 and D272 which were obtained after treatment of an *inos, ad*-5 (Y152) (adenine requiring) double mutant with 2-aminopurine.

Complementation was tested by superimposing standard loopfuls of conidia of each of the *leu*-2 mutants on agar slants containing synthetic medium supplemented with inositol. Adenine was added whenever one of the strains required it for growth. Complementation was scored after seven days incubation at 30°C. Growth was detected within 24 hr after mixing strains which

yielded vigorous heterokaryons. Most weak responses were not detectable until three days had elapsed and negative tests were often held for three or more weeks without noticeable growth.

All mutant strains used in this study that were not direct derivatives of *inos* 89601 were obtained in the 89601 genetic background after five successive backcrosses.

Growth rates were measured by the method of Ryan *et al.*[8] at 30 ± 2°C.

*Results.*—The determination of the complementation pattern of 158 mutants was simplified by testing all mutants against a relatively small set of *leu*-2 mutants. The set comprised: D6, D15, D18, D19, D22, D23, D28, D40, D50, D61, D69, D71, and R86. Almost half (78) of the mutants obtained complemented one or more of the test strains. These were subsequently tested in all pairwise combinations.

The results are summarized in Figure 1. Complementation patterns of all quali-

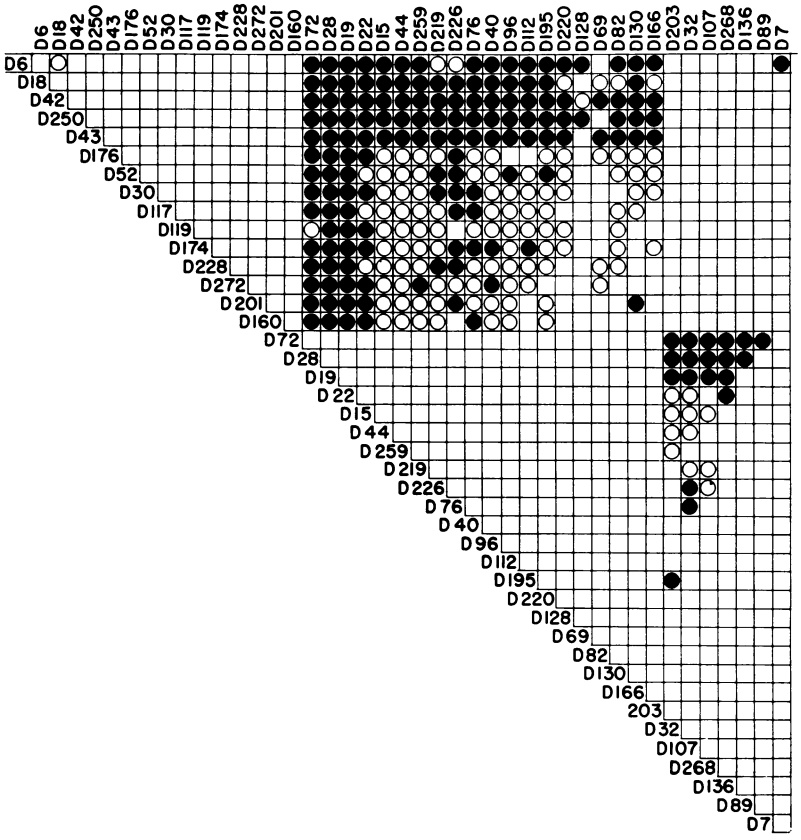


FIG. 1.—Results of pairwise complementation tests of *leu*-2-mutants. Good growth; ●; poor growth, ○; no growth, blank.

tatively different mutants are presented. Groups of mutants sharing similar patterns are referred to by that member of the group with the lowest numerical designation. The number of mutants comprising the group is noted in parentheses in Figure 2. Quantitative variation in response is frequent among members of these groups, but for the sake of simplicity only the pattern of the specifically listed mutant is given. The topographical projection of the complementation patterns is presented

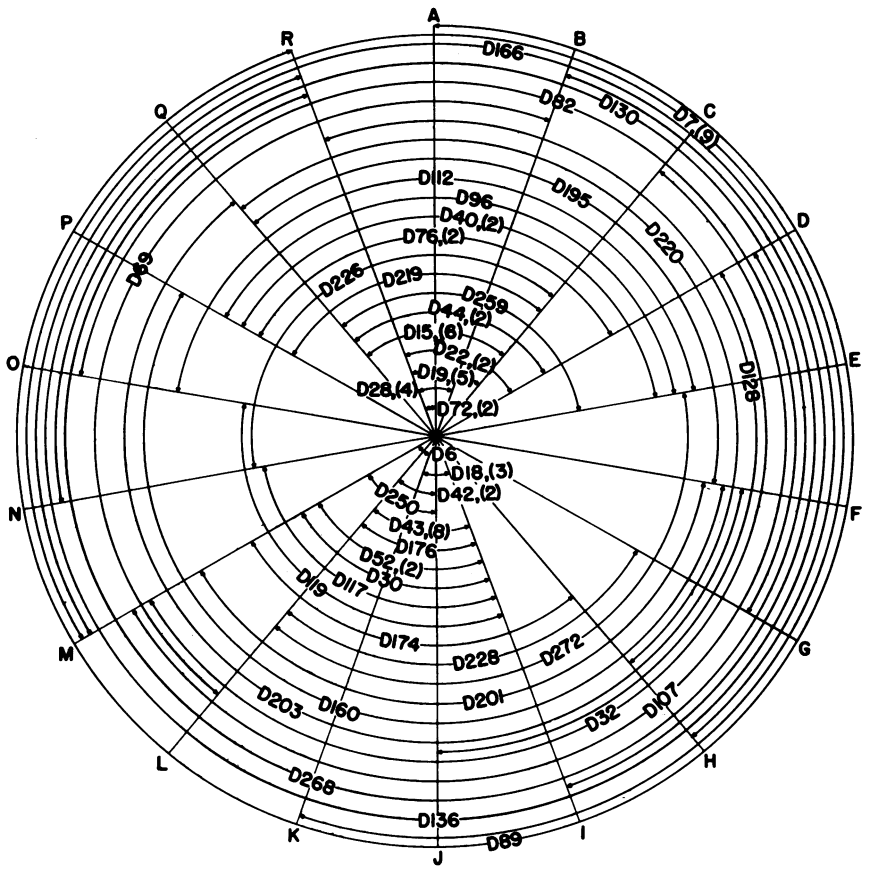


FIG. 2.—Complementation map of *leu*-2 mutants.

in Figure 2. The important feature of the map is that it is linear, continuous, and overlapping. The simplest geometrical representation is circular but no specific geometry other than linear continuity is implied.

Figures 1 and 2 include the complementation patterns of nine mutants involved in six anomalous tests (D176 by D96, D176 by D112, D201 by D112, D201 by D130, D220 by D117, and D30 by D82). The negative tests have been confirmed with "forced" heterokaryotic conidia. It should be noted that four of the combinations involve three mutants, each of which is a member of two anomalous pairs. The aberrant tests indicate the involvement of specific interactions between altered gene products which cannot be mapped in a simple linear array.

*Biochemistry.*—The biosynthesis of leucine in both *Neurospora* and *Salmonella* has been recently elucidated.[9, 10] The *leu*-4 locus of *Neurospora* controls the synthesis of the enzyme that catalyzes the synthesis of β-carboxy-β-hydroxyisocaproate by condensation of α-ketoisovalerate and acetyl coenzyme A. *Leu*-1 has been shown to control the synthesis of the enzyme or one of the enzymes involved in the formation of α-ketoisocaproate, the keto analogue of leucine, by oxidative decarboxylation of α-hydroxy-β-carboxyisocaproate.

The isomerization of β-carboxy-β-hydroxyisocaproate to α-hydroxy-β-carboxyisocaproate is of special importance. This reaction is analogous to the aconitase catalyzed conversion of citrate to isocitrate. As in the case of citrate isomerization, the production of an unsaturated compound

in equilibrium with both isomers can be demonstrated. Citrate isomerization, however, does not involve aconitate as a true free intermediate.[11] Evidence thus far obtained suggests that the isomerization of β-carboxy-β-hydroxyisocaproate to α-hydroxy-β-carboxyisocaproate is catalyzed by a single enzyme, an isomerase.[12] A brief summary of the available information is as follows: (1) the interconversion of β-carboxy-β-hydroxyisocaproate and α-hydroxy-β-carboxyisocaproate does not occur in the absence of the formation of an unsaturated compound (identified by absorption at 235 mμ). (2) The activity ratios of the conversions of β-carboxy-β-hydroxyisocaproate and α-hydroxy-β-carboxyisocaproate to the unsaturated compound remain constant through a 30-fold purification involving ammonium sulfate fractionation and chromatography on hydroxylapatite. (3) Thermal inactivation rates of both reactions are identical. (4) Both reactions are inhibited proportionally by a number of inhibitors.

*Leu*-3 and *leu*-2 mutants are deficient in the isomerase and all *leu*-2 and *leu*-3 mutants accumulate β-carboxy-β-hydroxyisocaproate in their growth medium, while *leu*-1 mutants, which are deficient in the enzyme or enzymes which convert α-hydroxy-β-carboxyisocaproate to α-ketoisocaproate, accumulate both α-hydroxy-β-carboxyisocaproate and β-carboxy-β-hydroxyisocaproate. No accumulation of the unsaturated compound has been observed in the growth medium of any of the mutants examined. Thus, it seems that complementation between *leu*-2 mutants involves the production of an enzyme whose total synthesis is controlled by two separate unlinked genes (*leu*-2 is on linkage group IV, leu-3 is on linkage group I[6]). Since *leu*-2 mutants complement *leu*-3 mutants and the heterokaryons grow at rates not too different from *leu*-1 by *leu*-2, and *leu*-4 by *leu*-2 heterokaryons, it seems unlikely that *leu*-2 or *leu*-3 is responsible for the production of a specific diffusable inhibitor or repressor.

*Theoretical.*—A plausible assumption is that the isomerase is at least a dimer consisting of two nonidentical polypeptide chains: an α chain, coded by the *leu*-2 gene and a β chain coded by the *leu*-3 gene. The enzyme phenotype of *leu*-2 mutants would be $\alpha^x\beta$ and that of *leu*-3 mutants $\alpha\beta^x$, where x denotes any mutationally determined structural alteration. If we assume that complementation does not involve protein fragments but rather an association of complete peptide chains, a simple model for complementation interactions between proteins produced by *leu*-2 mutants 1 and 2 would be the formation of an $\alpha^1\alpha^2\beta\beta$ tetramer. Such a model is analogous to the structure of hemoglobin which is a tetramer consisting of two peptide chains whose synthesis is controlled by two separate and distinct genes.[13] This model accounts for good complementation between *leu*-2 mutants (producing good β chains) and *leu*-3 mutants (producing good α chains), for, at least 6 per cent of the enzyme produced would be normal and 50 per cent of the hybrid enzyme molecules produced would have at least one normal α and one normal β chain (many of which should be enzymatically active as a consequence of complementation of a defective polypeptide by a normal one).

If the above considerations are reasonably close to reality, a striking difference should be observed in the effectiveness of complementation between *leu*-2 mutants if the production of normal β chains is restricted by the introduction of a *leu*-3 mutation into the genome of one of the complementing *leu*-2 pairs. Introducing a defective β chain should decrease the number of $\alpha^1\alpha^2\beta\beta$ tetramers from 50 per cent in the absence of a defective β chain gene to 12.5 per cent in the presence of a defective β chain gene if the polypeptides were synthesized at a normal rate and their assortment were random. Furthermore, Woodward *et al.*[14] have observed that the maximum adenylosuccinase activity in heterokaryons formed between complementing alleles is about 25 per cent of wild-type activity. It seems, therefore, more realistic to assume that the most efficient α chain hybrid would not have much more than 50 per cent of the activity of normal α chain pairs. The activity

expected from $\alpha^1\alpha^2\beta\beta$ tetramers in heterokaryons formed between a *leu*-2 mutant and a *leu*-2, *leu*-3 double mutant would be about 6 per cent.

If random assortment of peptide chains is assumed to occur, approximately 13 per cent of the tetramers will be $\alpha$ chain hybrids with normal $\beta$ chains and 25 per cent will be double hybrids. Very little can be predicted about the double hybrid combination. There is no *a priori* reason to assume that any particular $\alpha^1\alpha^2\beta^x\beta$ tetramer will not be functional. However, two parameters must be considered, the nature of the particular defects in both of the $\alpha$ chains and the defect in the $\beta$ chain. It may be that defective complementing $\alpha$ pairs present some conformational problem in the polymerization process with $\beta^x$ chains. Furthermore, if polymerization occurs, it seems reasonable to expect that most $\alpha^1\alpha^2\beta^x\beta$ tetramers would be relatively inactive because of structural limitations imposed by the complementary $\alpha$ chain defects.

The oversimplified model presented suggests that the complementation efficiency of *leu*-2 mutants would be very much reduced, for the most part, when heterokaryons are formed between a *leu*-2, *leu*-3 double mutant and a set of *leu*-2 mutants. It should be pointed out that no dramatic reduction in complementation efficiency would be expected under the following circumstances: (1) *leu*-2 and *leu*-3 determined the structure of two different enzymes, (2) complementation involved the recombination of protein fragments to produce a completely normal $\alpha$ polypeptide chain. Such a structure should combine with normal $\beta$ chains 50 per cent of the time, and (3) if recombination of the protein forming system were responsible for the formation of normal $\alpha$ chains.

The results of a series of complementation tests between a *leu*-2, *leu*-3 double mutant and a series of *leu*-2 mutants are presented in Table 1. The *leu*-2, *leu*-3 double mutant employed in this experiment consisted of the R86 *leu*-2 mutation (a member of the D19 group) which complemented efficiently all mutants not overlapping region R to C in Figure 2, and R156, the least leaky of a small set of *leu*-3 mutants among which no complementation has been observed. The complementation pattern obtained from tests of the *leu*-2, *leu*-3 double mutant was compared with the pattern obtained for the same set of *leu*-2 mutants tested with the *leu*-2 (R86) single mutant, a *leu*-2 (R86), *leu*-1 (33757) double mutant and *leu*-2 (R86), *leu*-4 (D229) double mutant. The *leu*-2, *leu*-1 and *leu*-2, *leu*-4 double mutants were used in order to rule out the possible involvement of unexpected metabolic influences on the complementation process.

The qualitative complementation tests presented in Table 1 indicate a sharp distinction between the *leu*-2 mutants which complement the *leu*-2, *leu*-3 double mutant. Only mutants in the D42 and D43 groups and mutants D250 and D256 complement with the efficiency observed in simple inter-allelic tests. The remainder of the *leu*-2 mutants that were positive in combination with the R86 single mutant, the *leu*-2, *leu*-1 (R86, 33757), and the *leu*-2, *leu*-4 (R86, D229) double mutants, yielded only trace amounts of growth with the *leu*-2, *leu*-3 double mutant. The only exception was D107, which was negative. In addition, a clear distinction could be made between the response of D64 and D183 and that displayed by D43, D68, and D118, members of a group of mutants which could not be distinguished on the basis of simple pairwise complementation tests. All *leu*-2 mutants which did not complement the R86 single mutant, failed to complement the *leu*-2, *leu*-3 double

TABLE 1

THE EFFECT OF AN ALTERED *Leu*-3 GENE ON COMPLEMENTATION OF *Leu*-2 ALLELES

| | Qualitative Growth Responses of Heterokaryons | | | |
|---|---|---|---|---|
| *Leu-2* mutant | *Leu-2, Leu-3* (R86), (R156) | *Leu-2, Leu-1* (R86), (33757) | *Leu-2, Leu-4* (R86), (D229) | *Leu-2* (R86) |
| D6 | Trace | + | + | + |
| D18 | Trace | + | + | + |
| D142 | Trace | + | + | + |
| D256 | + | + | + | + |
| D42 | + | + | + | + |
| D161 | + | + | + | + |
| D250 | + | + | + | + |
| D52 | Trace | + | + | + |
| D180 | Trace | + | + | + |
| D43 | + | + | + | + |
| D64 | Trace | + | + | + |
| D68 | + | + | + | + |
| D118 | + | + | + | + |
| D183 | Trace | + | + | + |
| D104 | Trace | + | + | + |
| D120 | Trace | + | + | + |
| D129 | Trace | + | + | + |
| D119 | Trace | + | + | + |
| D30 | Trace | + | + | + |
| D107 | − | + | + | + |
| D174 | Trace | + | + | + |
| D228 | Trace | + | + | + |
| D176 | Trace | + | + | + |
| D117 | Trace | + | + | + |
| D201 | Trace | + | + | + |
| D32 | Trace | + | + | + |

Good growth within 24–48 hr, +; complementation barely detectable at the end of 7 days and growth only slight after 14 days incubation at 30°C, trace; no growth, −. Brackets signify mutants with similar pairwise complementation patterns.

mutant.

Quantitative growth determinations of several heterokaryons formed between *leu*-2 mutants and the *leu*-2, *leu*-3 double mutant are presented in Figure 3. Measurements of growth of the corresponding *leu*-2 complementing pairs are also presented. The quantitative determinations support the conclusions derived from the qualitative observations and indicate the sharp distinction between the complementation efficiency of the *leu*-2 mutants tested in pairwise combinations and the corresponding tests performed with the *leu*-2, *leu*-3 double mutant. However, the growth determinations of heterokaryons formed between *leu*-2 single mutants and R86 revealed that the *leu*-2 mutants which poorly complemented the *leu*-2, *leu*-3 double mutant had slightly slower growth rates (2.8 mm/hr) in heterokaryons with R86 than those mutants which complemented efficiently (3.25 mm/hr). The growth rates of the *leu*-2, *leu*-1, the *leu*-2, *leu*-4 and the *leu*-2, *leu*-3 double mutants, as well as those of the *leu*-2, and *leu*-3 single mutants varied between 3.25 and 4 mm per hr on medium containing 150 μgm leucine per ml. Heterokaryons formed between several heterologous mutants and the single and double *leu* mutants grew at 3.25 to 4 mm per hr on minimal medium.

These results show clearly that the introduction of a mutation in the *leu*-3 locus has a profound effect on the complementation efficiency of most of the *leu*-2 alleles. The depression of complementation efficiency in *leu*-2 by *leu*-2, *leu*-3 heterokaryons seems to be associated with those leu-2 mutations which in combination with R86, form heterokaryons which grow at a slightly reduced rate. Hence, on the basis of the model presented, it seems probable that those $\alpha$ chain alterations

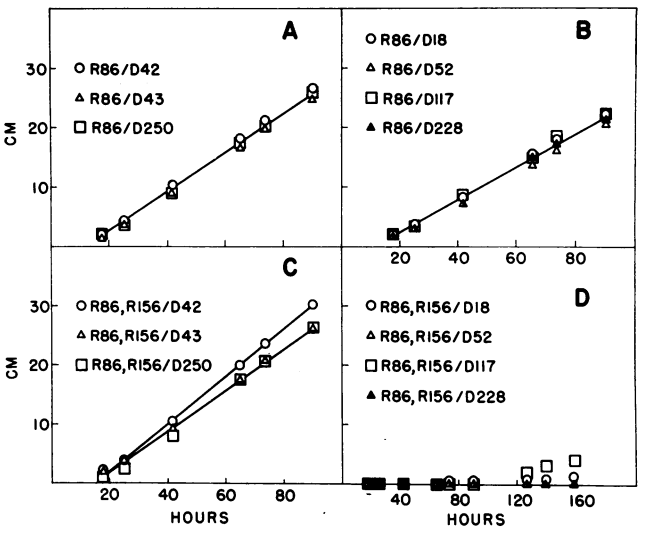


FIG. 3.—Growth progression as a function of time displayed by several *leu*-2 heterokaryons. Heterokaryons formed between *leu*-2 (R86) and *leu*-2 mutants, curves A and B. Heterokaryons formed between the *leu*-2 (R86), *leu*-3 (R156) double mutant and *leu*-2 mutants, curves C and D. Growth was initiated with conidial inocula obtained from heterokaryons that grew well on slants and with heterokaryotic mycelia from heterokaryons that grew poorly.

which yield $\alpha^1\alpha^2\beta\beta$ tetramers with reduced activity yield relatively inactive $\alpha^1\alpha^2\beta^x\beta$ molecules. However, it is not possible, on the basis of the information available, to rule out a specific inhibition of the polymerization of specifically altered $\alpha$ chains by $\beta^x$ chains.

Variations in nuclear ratios that may ensue after growth is initiated could exaggerate differences in the growth response of different heterokaryons formed between *leu*-2 mutants and the *leu*-2, *leu*-3 double mutant. Thus far it has been impossible to check this point adequately because of the failure to obtain sufficient conidia for analysis of the heterokaryons that grow slowly, without first encountering reversions, etc. However, no evidence for sharp departures from the equal input nuclear ratios has been observed in the limited number of analyses done on the heterokaryons which grew rapidly.

*Discussion.*—The data presented imply that the isomerase consists of two different polypeptide chains, $\alpha$ and $\beta$, each coded by separate nonlinked genes, *leu*-2 and *leu*-3. The interactions of specifically altered $\alpha$ chains observed in the heterokaryons formed between $\alpha$ chain mutants and the double mutant containing both altered $\alpha$ and $\beta$ chains indicate that the $\alpha^1\alpha^2$ complex necessary for functional activity does not restore the exact structural configuration of the original $\alpha\alpha$ structure. This is in agreement with the observations in other systems that the enzymes produced by complementation are different from the normal enzymes[4] and, together with the results obtained here, renders untenable certain hypotheses implying that complementation results from the free association of normal fragments of polypeptide chains in the formation of an intact polypeptide. It should be pointed out that certain hypotheses involving the association of incomplete polypeptides in nonpeptidic linkages in a manner analogous to the association of fragments of ribonuclease produced by subtilisin digestion may be tenable.[15] However, rather

powerful arguments have been presented suggesting that peptide fragments generally are not produced as a consequence of gene mutation (Crick *et al.*[16]).

The sharp discontinuity in the growth responses of the several heterokaryons formed between *leu*-2 mutants and the *leu*-2, *leu*-3 double mutant raises some problems relevant to the proposed structure of the isomerase. However, it is important to note that the *leu*-3 mutant, R156, when grown on limiting leucine (derepression?) can produce about 5 per cent of the isomerase activity found in the wild-type strain. This mutant does not grow in the absence of exogenous leucine. It seems probable that metabolic control mechanisms prevent growth when the ability to produce active enzyme falls below some limit. Therefore, the discontinuity in growth responses may simply result from a reduction in enzyme activity to a level reasonably close to the 6 per cent predicted on the basis of the interaction of four subunits.

Although the above suggests that a tetrameric structure is not inconsistent with the data presented, larger aggregates are not excluded. Only a direct structural analysis of the enzyme can resolve this question. However, it is important to point out that the mechanism of polymerization is of vital importance to the interpretation of the complementation interaction. One would like to know whether an association of $\alpha$ and $\beta$ chains occurs prior to polymerization of $\alpha\beta$ duplexes or whether $\alpha\alpha$ and $\beta\beta$ associations are independent of each other.

*Summary.*—An analysis is presented of the complementation behavior of a large group of *leu*-2 mutants. The complementation map derived is linear, overlapping, and continuous. Evidence is presented that indicates that the polypeptide whose structure is determined by the *leu*-2 gene is one of at least two different peptide structural units of an enzyme which catalyzes the isomerization of $\beta$-carboxy-$\beta$-hydroxyisocaproate and $\alpha$-hydroxy-$\beta$-carboxyisocaproate. An hypothesis is presented relating complementation between *leu*-2 mutants to a protein-protein interaction in the formation of a polymer consisting of $\alpha$ chains coded by the *leu*-2 gene and $\beta$ chains coded by the *leu*-3 gene which is at an unlinked locus. The complementation behavior of a group of *leu*-2 mutants in combination with a *leu*-2, *leu*-3 double mutant is consistent with the notion that the isomerase is a mixed polymer consisting of at least two $\alpha$ and two $\beta$ chains.

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# *SPONTANEOUS CHROMOSOME ABERRATIONS IN DROSOPHILA ROBUSTA**

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Although spontaneous chromosomal aberrations are generally rare, high aberration rates have been reported from time to time in corn,[1-4] *Tradescantia*,[5] and *Drosophila melanogaster*.[6, 7] Recently more than forty new chromosome aberrations have appeared in *Drosophila robusta* in this laboratory. This outburst is made particularly interesting by evidence that the factors responsible involve a maternal effect.

*Materials and Methods.*—Carson and Stalker[8] described the widespread gene arrangements of *Drosophila robusta* Sturtevant, a species inhabiting the deciduous woods of eastern North America. Since 1947, the author has been studying chromosomal variations in wild population of this species. The analyses[9-11] have endeavored to determine the complete karyotypes of collected adults by progeny testing. Collected males and, since 1950, despermed females as well have been crossed to stock flies homozygous for known gene arrangements, and 8–10 larval salivary gland smears from each cross have been examined. Many of the analyses of captured females were continued further for crossover studies.[12] Some of the wild strains were inbred to provide strains for population cage studies.[9] The most recent experimental population studies (unpublished) have included analyses of adult samples similar to those described above for the wild populations.

The homozygous stock used in any progeny test was chosen in a routine and essentially random way, governed only by the availability of the stock, the current "health" of its cultures, etc. Several stocks were commonly used in the crosses made on any given day, and the crosses using the various stocks were not segregated in the containers or incubators.

The stocks regularly contributing virgins and males for the progeny tests and their histories follow:

1. $ST_y$: homozygous for all the "Standard" gene arrangements; developed by the author in 1948 by inbreeding descendants of a female collected in Tibbetts Brook Park, Yonkers, Westchester County, New York. The stock has been lost several times in this laboratory but kindly replenished from a duplicate maintained by H. L. Carson. About one year ago, a duplicate substrain, dubbed "$ST_y$-B," was established here, and the continuation of the original stock was named "$ST_y$-A."

2. $ST_{pl}$-A and $ST_{pl}$-B: duplicate substrains of a vigorous stock obtained from the cross of a despermed 1960 Swarthmore, Delaware County, Pennsylvania female and $ST_y$ males when she proved to be homozygous Standard in all arms.