ORGANIZATION OF THE RUDIMENTARY WING LOCUS IN
DROSOPHILA MELANOGASTER.
I. THE ISOLATION AND PARTIAL CHARACTERIZATION OF MUTANTS
INDUCED WITH ETHYL METHANESULFONATE, ICR-170 AND X RAYS

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ABSTRACT

New rudimentary (r) mutants have been isolated following mutagenesis
with ethyl methanesulfonate (rLE), ICR-170 (rL1) and X rays (rLX). From
wing phenotype measurements on homoallelic females, it has been shown
that the rLE mutant series includes several leaky alleles, as well as alleles
that produce moderate and strong r phenotypes. All of the tested rL1 alleles
yielded strong r phenotypes in homoallelic females, whereas the rLX series
was found to include both moderate and strong alleles. Based on allele
complementation for the wing phenotype, it was found that all three mutant
series include both complementing and noncomplementing alleles, but the
relative frequencies of these two types of alleles differ considerably among
the three series. Complementing alleles comprise most of the rLE mutant
series (19 of 25) and almost one-half of the rLX series (five of 12), while
only one of 16 rL1 mutants is a complementing allele. Data from enzyme
assays of mutants mostly support the direct correlation of genetic comple-
mentation units with the activities of the first three enzymes in the de
novo pyrimidine biosynthetic pathway. All of these findings are discussed
in light of evidence that these three enzymes are contained within a tri-
enzyme complex in animals. We conclude that the available genetic evidence
supports the contention that the trienzyme complex is encoded by a single
mRNA.

IN Drosophila melanogaster, a small genetic region, the rudimentary wing
(r) locus, encodes the first three enzymes of the pathway for de novo pyrimi-
dine biosynthesis: glutamine-utilizing carbamyl phosphate synthetase (CPSase;
EC 2.7.2.9), aspartate transcarbamylase (ATCase; EC 2.1.3.2) and dihydro-
orotase (DHOase; EC 3.5.2.3) (Norby 1970, 1973; Jarry and Falk 1974;
Rawls and Fristrom 1975; Rawls 1979b). In animals, these enzymes are con-
tained in a trienzyme complex (Jones 1972; Mori and Tatibana 1975; Cole-

Numerous r mutants have been described, some of which exhibit allelic com-
plementation, while others are completely noncomplementing (Fahmy and
Fahmy 1959; Green 1963; Carlson 1971). Complementing mutants are lo-
cated throughout the genetic fine-structure map of the r locus (Green 1963;
Carlson 1971) and lack one or more of the three activities of the CPSase-
ATCase-DHOase complex (Norby 1973; Jarry and Falk 1974; Rawls and Fristom 1975; Fausto-Sterling 1977). These studies generally indicate that complementing mutants lacking ATCase activity are concentrated near the centromere-proximal end of the \( r \) locus, those lacking CPSase occupy the center, and those lacking DHOase map near the centromere-distal end.

Noncomplementing \( r \) mutants uniformly lack all three enzyme activities, and of the noncomplementing mutants examined by Carlson (1971), most mapped near the centromere-proximal end of the locus. This apparent concentration of noncomplementing mutants within the presumed ATCase-specifying region suggests that some might be polarity mutants resulting either from mutations within a cis-acting regulatory region or from frameshift and nonsense mutations within a polypeptide-encoding region of the locus. The latter would imply that the CPSase-ATCase-DHOase complex is translated from a single mRNA. Another possible explanation for noncomplementing mutants is that they include a variety of missense mutations that result in conformational changes within the complex that effectively inactivate the entire complex. Unfortunately, detailed genetic analyses of the \( r \) locus preceded the discovery of \( r \) function, and many of the more interesting \( r \) alleles have been lost.

In order to study the precise relationships between \( r \) genetic structure and the organization of the CPSase-ATCase-DHOase complex, we have collected a variety of new \( r \) mutants, starting with a single progenitor wild-type allele. Mutants have been obtained using ethyl methanesulfonate (EMS), the monofunctional quinacrine mustard ICR-170, as well as X rays. This paper reports the recovery of these mutants, their complementation behavior, and their enzymatic phenotypes. We have obtained decidedly different types of \( r \) mutants with these three mutagens in a manner consistent with the premise that the CPSase-ATCase-DHOase complex is translated from a single mRNA.

**MATERIALS AND METHODS**

**Media and growth conditions:** Flies were routinely cultured on yeast-sucrose-glucose-cornmeal medium (Lewis 1960). Where indicated, the basic medium was supplemented with crude Torula yeast RNA (Sigma Chemical Co.) to a concentration of 1% (w/v). Flies used for enzyme assays were cultured at 25° ± 0.5°, but all other flies were grown at room temperature (22° ± 2°).

**Wing phenotype measurements:** All measurements were performed on one- to five-day-old adult females that were grown in uncrowded cultures containing standard medium. To describe wing phenotypic severities, we devised a numerical scoring system based upon the wing phenotypes described by Green (1963). Type 1 is a wing that is visibly indistinguishable from normal. Type 2 is a normally shaped wing that has irregularly spaced marginal bristles. In Type 3, the wing shape is noticeably altered, but the posterior tip of the wing extends beyond the tip of the abdomen. Type 4 is a more severely truncated wing that does not extend posteriorly beyond the tip of the abdomen. Type 5 is essentially a Type 4 wing that is severely wrinkled and/or vesiculated. For each genotype examined, a minimum of ten females were scored and a simple arithmetic mean was obtained from the scores of individual females.

**Allelic complementation:** Allele complementation was measured as the degree of restoration of normal wing morphology in \( trans \) heteroallelic females. From appropriate \( inter se \) crosses,
heteroallelic females were obtained and scored for the \( r \) wing phenotype according to the system described above. The results are sometimes abbreviated as complete complementation (+), in which all heteroallelic flies were Type 1; partial complementation (±), in which a mixture of Type 1 and Type 2 flies, or Type 2 flies alone, was observed; or noncomplementation (−), which included Type 3, 4 and/or 5 wings.

**Genetic markers and strains:** The mutants \( r^{54c} \), \( r^{531} \) and \( r^{3719} \) were described by Fahmy and Fahmy (1959) and Carlson (1971), while \( r^{39k} \) and \( r^9 \) were described by Green (1963), Lindsley and Grell (1968) and Carlson (1971). The four alleles, \( r^{54c} \), \( r^{531} \), \( r^{39k} \) and \( r^{3719} \), are mutually complementing (Figure 1), and virtually all \( r \) mutants fail to complement at least one of these alleles (Carlson 1971; Rawls, unpublished observations). The mutant \( r^{71286} \) arose spontaneously and was isolated by D. Mohler (personal communication). It displays an unusual complementation pattern (Figure 1). Together with \( r^9 \), \( r^{71288} \) has been extensively employed in screens for new \( r \) mutants, since these two alleles are mutually complementary (Figure 1), and heteroallelic females exhibit normal fecundity and fertility, yet most potential new \( r \) mutants should fail to complement either \( r^9 \) or \( r^{71286} \).

G. Lefèvre isolated \( r^{70286} \) following X-ray mutagenesis; it is a noncomplementing allele (Figure 1) that is associated with an X-chromosome inversion, \( In(1) r^{70286} \), with breakpoints in salivary gland chromosome regions 7B and 15A1–2 (G. Lefèvre, personal communication).

![Table](image)

**Figure 1.—** Allele complementation among old \( r \) mutants. Wing phenotypes were measured on *trans* heteroallelic females, as described in the MATERIALS AND METHODS. Results are presented as the range of phenotypes observed among the minimum of ten females examined. For noncomplementing heteroallelic combinations (*i.e.*, other than Type 1 wings were observed), the mean phenotypic score obtained from the individual female scores is presented in parentheses.
$Df(1) r^{D17}$ is a small X-ray-induced deficiency isolated by M. M. Green; it is cytologically deficient for chromosome region 15A1–5 and is apparently deleted for the $r$ locus and one or more adjacent recessive lethal loci (M. M. Green and G. Lefevre, personal communications). $Df(1) r^{D17}$ always elicits the most extreme phenotype possible from the particular allele with which it is tested (Figure 1), in agreement with the cytogenetic evidence that this deficiency involves a physical deletion of the $r$ locus. $Dp(1;4) r^+ f^+$ is a small segment of the $X$ chromosome, including the $r^+$ locus, that is attached to a chromosome 4 centromere (Green 1963). The balancer chromosome designated $FM0$ is actually $In(f) sc^8 + dli - 49, y^{2nd} sc^8 w c m^2 v f B$, a male-viable and recessive female-sterile chromosome that was constructed by D. Mohler (personal communication). Markers are described in Lindsley and Grell (1968).

All new mutants were induced in a chromosome bearing the markers $v tc r^{L+1} f$. The wild-type isoallele $r^{L+1}$ has been maintained in this laboratory since its isolation in a solitary male in 1976. The marker $tc$ lies 3.29 map units distal to $r$, while $f$ lies 1.27 map units proximal (Carlson 1971).

Mutagen treatments: All mutagenesis was performed on mixtures of one- to four-day-old $v tc r^{L+1} f$ males. EMS treatment was patterned after the method of Lewis and Bacher (1968) by feeding males a 2% solution of sucrose containing 50 mm of EMS (Sigma Chemical Co.). After 20 hr on the mutagen, males were removed to standard medium for 24 hr. They were then mated with $r^{9} r^{71326}$ females for four days on RNA-enriched medium, after which time the males were discarded and the inseminated females were transferred to fresh medium. After four additional days, the females were again transferred to fresh medium, then discarded four days later.

ICR–170 mutagenesis was performed using the method of Carlson and Oster (1962); each male received an intra-abdominal injection of a fresh solution of 0.7% saline containing 0.1% ICR–170 (a gift from H. Creech and his associates at the Institute for Cancer Research, Philadelphia). Other procedures were exactly as described for the EMS mutagenesis, except that after injection, males as well as females were maintained in darkness throughout their period in culture.

For X-ray mutagenesis, males were subjected to 5000 rads from a Vanguard Orthovoltage X-ray machine. In contrast to the EMS and ICR–170 screens, males were mated with females immediately after irradiation. After four days, the males were discarded and females were transferred twice to fresh cultures at four-day intervals, as described above.

A simple test for sex-linked lethals was routinely performed to measure the effectiveness of mutagenesis. Mutagenized males were mated with attached-X, $C(1) DX,y f$ females rather than $r^{9} r^{71326}$ females, but otherwise all procedures were as described above. Male and female progeny were counted and the resulting sex ratio was corrected for marker and background viability differences by comparison with the sex ratio obtained from cultures sired by nonmutagenized males (i.e., the sex ratio from mutagenized-male cultures was divided by the ratio in control cultures). This corrected sex-ratio value served as a coarse index of the frequency of autonomous sex-linked produced by the mutagen treatment.

Isolation of new mutants: The protocol for mutant detection and isolation is shown in Figure 2. For each screen, approximately 400 mutagenized males and a comparable number of $r^{9} r^{71326}$ females were mated at a density of 12 pairs of flies per half-pint bottle (Cross 1 in Figure 2). Female progeny from the initial and transfer cultures were visually examined and each phenotypically $r$-wing female was individually mated with several $FM0$ males in a shell vial (Cross 2). Among the offspring of each vial, phenotypically $tc r f$ males were sought and, if they occurred, these males were used to establish a stock of the new mutation. From each Cross 2 vial that produced no $tc r f$ males, up to ten phenotypically $f B$ females (i.e., $tc f/ FM0$ heterozygotes) were collected and each female was placed in a vial with several $FM0$ males (Cross 3). Each of these subcultures was examined to determine whether an $r$ mutation was present within the $tc f$ chromosome and/or whether a lethal mutation allelic or closely linked to $r$ was present within the $tc f$ chromosome. These questions were
Cross 1: \( \text{mutagen} \) v to \( y^{+2} f / Y \) 

Examine female offspring and select visibly \( r \) individuals

Cross 2: \( q \) v to \( r^{\text{new}} f / r^{9} \) or \( \text{71j86} \)

if no to \( r f / Y \) males emerge, select and individually subculture up to ten phenotypically \( f \) \( b \) females

Cross 3: \( q \) to \( r^{2} f / \text{POM} \)

if no to \( r f / Y \) males emerge from any subcultures, from subcultures in which no to \( f / Y \) males emerged cross a few phenotypically \( f \) \( b \) females to \( Df(1) \text{rD17}, v f / Dp(1;4) r^{+} f^{+} / Y \) males to test for a lethal and/or \( r^{-} \).

establish stock

FIGURE 2.—Protocol for isolation of new \( r \) mutants. Details are in MATERIALS AND METHODS.

resolved either by the emergence of \( tc r^{+} f \) or \( tc r f \) males within the subcultures or by further appropriate crosses of heterozygous females from the subcultures to \( Df(1) \text{rD17}, v f / Dp(1;4) r^{+} f^{+} \) males.

Each putative \( r \) mutation was identified as an allele of \( r \) by its failure to complement \( r^{70b26} \). New alleles are assigned superscript designations that identify their source (\( L \) for Lexington), mutagen used (\( E \) for EMS; \( X \) for X rays; \( I \) for ICR-170), and a number that approximates the order in which mutants arose in the various screens employing each mutagen.

Enzyme activity determinations: Each sample consisted of a mixture of adult male flies ranging in age from one to four days post-emergence. Flies were ground in glass Ten Broeck tissue grinders at a ratio of 0.1 g per ml of buffer (50 mM potassium phosphate, \( \text{pH} 7.0 \); 10% glycerol; 10 mM 2-mercaptoethanol). After centrifugation for one hr at 30,000g, 0.2 ml of the supernatant was placed on a 0.8 ml bed of Bio-Gel P-30 that was equilibrated with buffer. The column was developed and the void volume fraction was used in enzyme and protein assays within two hr.

Glutamine-dependent CPSase and protein were assayed as described by \textsc{Rawls} (1979b). ATCase was determined radiochemically using reaction mixtures (25 \( \mu l \)) that contained Tris-Cl buffer (0.1 M, \( \text{pH} 9.2 \)), dilithium carbamyl phosphate (5 mM), \( L-[^{14}\text{C}] \)-aspartate (10 mM, 0.5 \( \mu \text{Ci}/\mu \text{mol}, \text{Schwarz/Mann} \)), and 15 \( \mu l \) of enzyme sample. After one hr at 30\(^{\circ}\), the reaction was terminated by adding 5\( \mu l \) of 10 N formic acid. Radioactive carbamylaspartate was separated by chromatography on DEAE-cellulose paper (\textsc{Herrman, Dunn} and \textsc{Schmidt} 1973), and radioactivity within the carbamylaspartate spot in each chromatogram was counted by liquid scintillation spectrometry (\textsc{Rawls} 1979a).

DHOase was measured in reaction mixtures (25 \( \mu l \)) that contained Tris-Cl buffer (0.1 M, \( \text{pH} 8.0 \)), \( DL-[^{14}\text{C}] \)-dihydroorotase (1 mM, 5 \( \mu \text{Ci}/\mu \text{mol}, \text{New England Nuclear} \)), and 20 \( \mu l \) of enzyme sample. After incubation for 30 min at 30\(^{\circ}\), the mixtures were acidified and radioactive carbamylaspartate was separated and quantitated as described for the ATCase assay.
Enzyme samples from $r^{L+1}$ flies were prepared and assayed alongside mutant samples. For each enzyme sample, all enzyme activities were determined in replicate reaction mixtures, and a mean value was computed for each enzyme activity as picomoles of enzyme product formed per min per $\mu$g of protein. Each mutant specific-activity value obtained on a given day was normalized using the specific-activity value from the parallel $r^{L+1}$ sample, and mutant activity values are expressed as the percentage of $r^{L+1}$ activity. This normalization of mutant values considerably reduces variances in the data due to unavoidable day-to-day differences in enzyme preparation and assay.

RESULTS

Isolation of new $r$ mutants: In all screens for mutants (Figure 2), the criteria for the detection and isolation of mutations were identical, regardless of the particular mutagen used. Only flies showing unequivocally irregular spacing of the marginal wing bristles were scored as phenotypically $r$. This phenotype is highly specific for true $r$ mutants (Lindsley and Grell 1968), and it is easily distinguished from the variety of dominant mutations, as well as developmental and environmental malformations, that were sporadically observed among first-generation females in the screens. In the experiments described here, never was a female initially diagnosed as phenotypically $r$ that subsequently proved to have been expressing a nonallelic wing mutation. These considerations argue that little ambiguity is encountered in distinguishing putative $r$ mutants; yet, it is likely that females expressing extremely weak mutant phenotypes were sometimes undetected. The likelihood that such a female would go undetected was probably comparable for each screen, however, and any observed differences in the frequencies of such females in various screens probably reflect the diverse properties of the mutagens rather than differences in the protocols used to select mutants.

We have carried out a total of eight different screens using EMS, nine with ICR-170, and three with Xrays. For each mutagen, we have detected no substantial differences among the results of the various screens; therefore, the results of the screens have been pooled and are presented in Table 1. A large

<table>
<thead>
<tr>
<th>Mutagen</th>
<th>EMS</th>
<th>ICR-170</th>
<th>X rays</th>
<th>none†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of chromosomes examined‡</td>
<td>57,430</td>
<td>177,729</td>
<td>35,635</td>
<td>15,936</td>
</tr>
<tr>
<td>Number of $r$ females observed</td>
<td>147</td>
<td>134</td>
<td>27</td>
<td>1</td>
</tr>
<tr>
<td>Number of new mutants isolated</td>
<td>30</td>
<td>16</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Autonomous sex-linked lethal index‡</td>
<td>0.37</td>
<td>0.66</td>
<td>0.68</td>
<td>–</td>
</tr>
</tbody>
</table>

*Methods of mutagenesis are described in the MATERIALS AND METHODS section.
† Untreated males served as parents in the screen.
‡ Equals the number of first generation females examined in the screen (see Figure 1).
§ The corrected sex ratios observed among the offspring from crosses of mutagenized males and attached-X females, as described in MATERIALS AND METHODS.
number of first generation females expressed new r mutations mosaically. That is, one wing was phenotypically r and the other was normal. In the EMS and ICR-170 screens, such females were in the majority, as is common in mutagenesis with monofunctional alkylating mutagens (Lee 1976). All first-generation females showing r wings are included in Table 1, irrespective of whether they were visibly mosaic or "complete." In the EMS and ICR-170 screens, most of the "complete" as well as the mosaic females transmitted tc r+f chromosomes to their offspring, showing that their gonads contained nonmutant tissue. Details of the mosaicism encountered in these screens will be presented elsewhere.

EMS mutagenesis yielded the highest frequency of r mutations with 0.26% of all treated chromosomes containing apparent r mutations. One-fourth of the first generation r females were sterile, but the rest were fertile due to the inclusion of RNA in the medium on which they were grown (Bahn 1970). The majority of fertile r females transmitted only tc r+f chromosomes, being presumably mosaic flies. Consequently, only 30 rLE mutants were eventually isolated in stock cultures. Sometimes chromosomes bearing certain of these new mutants were inviable in hemizygous males, but in all cases the lethal proved separable from the new r mutation by recombination with a nonmutagenized v tc rL+rLE chromosome. Five rLE mutants were lost prior to the completion of complementation tests, leaving a total of 25 to be described in subsequent sections of this paper.

New r mutations were apparently present in 0.075% of the ICR-170-mutagenized chromosomes. Of the first generation r females, 25% were sterile and the frequency of mosaicism among the fertile females was high, leading to the recovery of only 16 rLrLE alleles. All of these mutants were separable from any linked lethals and all are described in subsequent sections of this paper.

Using Xrays, 0.076% of the mutagenized chromosomes contained apparent new r mutations. Among the first generation females, 24% were sterile, but few of the fertile females displayed any form of mosaicism. Therefore, the success rate in establishment of new rLX mutants was better and a total of 13 were established as stocks. The stock containing one of these mutants was poorly viable and was lost prior to the completion of complementation tests. Several suggestive aspects of this mutation were: (1) it failed to complement several r mutants; (2) it was lethal in hemizygous males and in females heterozygous for Df(1)rD77; (3) it produced a strong, dominant Minute phenotype in heterozygotes (Lindsley and Grell 1968). These observations indicate that this mutation was a large chromosome deficiency that lacked the r locus, a neighboring Minute, and, perhaps, other essential loci. The 12 other rLX mutants gave viable r phenotypes in Df(1)rD77 heterozygotes and most were separable from any linked lethals.

Phenotypic assays of new r mutants: The r wing phenotype was measured in females homoallelic for most of the new mutants and the results are presented in Figure 3. The entire range of r phenotypes was found among rLE alleles, with rLE3, rLE10 and rLE16 giving entirely normal wings in homoallelic females, while some of the other rLE alleles gave extreme phenotypes. Among the rLX mutants,
all yielded strong $r$ phenotypes in homoallelic females. Only a few of the $r^{LX}$ mutants were examined, but those examined presented a broader range of phenotypes than did $r^{LI}$ mutants.

We also examined all of the mutants listed in Figure 3 in females heterozygous for $Df(1)r^{DI7}$. Without exception, such heterozygotes exhibited wing phenotypes that were as extreme or more extreme than the phenotypes exhibited by mutant homoallelic females. For example, $r^{LE3}$, $r^{LE10}$ and $r^{LE18}$ all produced Type 2 wings in $Df(1)r^{DI7}$ heterozygotes, and all of the $r^{LI}$ alleles gave Type 4 and 5 wings, exclusively, in such females.

Many of the phenotypically strong $r$ mutants were poorly viable when cultured on standard medium either as homoallelic females or as $Df(1)r^{DI7}$ heterozygotes. In all cases, however, at least some of these females, as well as hemizygous mutant males, were obtained.

Complementation behaviors of new $r$ mutants: All new mutants were tested by observing wing phenotype complementation in trans heterozygotes bearing the new mutant and the tester alleles $r^{56c}$, $r^{50l}$, $r^{30h}$, and $r^{3719}$. These four alleles are mutually complementing alleles (Figure 1) and virtually all known $r$ mutants fail to complement at least one of them (Carlson 1971; Rawls, unpublished observations). Therefore, these four mutants represent the four major, unambiguous complementation units of $r$; we designate these units A, B, C and D. These units correspond to complementation units I, III, VI and VII, respectively, of Carlson (1971). Other $r$ mutants are conveniently described by their
complementation behaviors against $r^{54c}$, $r^{54l}$, $r^{30k}$, and $r^{3719}$. For example, $r^9$ fails to complement $r^{39k}$, yet it complements the other three tester alleles (Figure 1); thus, $r^9$ is designated $A+B+C^-D^+$. If $r^9$ had only partially complemented $r^{39k}$, it would have been assigned to complementation group $A+B+C^-D^+$.

The complementation behaviors of new $r$ mutants are represented in Figure 4. In general, the new mutants belong to complementation groups similar to those described previously (FAHMY and FAHMY 1959; GREEN 1963; CARLSON 1971). The largest single complementation group consists of $A^-B^-C^-D^-$ alleles, with $A+B+C^-D^+$ alleles comprising the second most common group. Most complementation tests yielded unambiguous wing phenotypes; that is, clearly mutant wings or entirely normal wings. However, partial complementation was detected among tests of mutants belonging to three complementation groups: $A+B+C^=D^+$ ($r^{LE3}$, $r^{LE10}$ and $r^{LE18}$), $A+B=C^-D^+$ ($r^{LE12}$ and $r^{LX12}$), and $A+B+C^=D^-$ ($r^{LE19}$ and $r^{LX3}$).

COMPLEMENTATION UNITS

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<td>LE35</td>
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FIGURE 4.—Allele complementation behavior of new $r$ mutants. New mutants were tested for allele complementation for the wing phenotype as described in MATERIALS AND METHODS. Failure to complement completely the tester alleles representing the four complementation units (Type 3, 4 or 5 wings is shown by a solid bar under the appropriate complementation unit. Partial complementation (Type 1 and 2 wings or Type 2 wings alone) is shown by a dashed bar. Complete complementation (Type 1 wings only) is shown by the absence of a bar.
The most striking feature of the data in Figure 4 is that the various mutant series contain distinctly different frequencies of complementing and noncomplementing alleles. The \( r^{LE} \) mutants include a wide variety of complementation groups, and only about one-fourth (six of 25) are \( A^{-}B^{-}C^{-}D^{-} \) alleles. However, all but one of the \( r^{LI} \) mutants are \( A^{-}B^{-}C^{-}D^{-} \) alleles (15 of 16). The exception, \( r^{LE8} \), is an \( A^{+}B^{+}C^{+}D^{-} \) allele. The frequencies of complementing and noncomplementing alleles in the \( r^{LE} \) series versus the \( r^{LI} \) series differ significantly (contingency \( \chi^2 = 16.31, p < 0.0001 \)). The \( r^{LX} \) mutants include a number of complementing mutants, but slightly more than half (seven of 12) are \( A^{-}B^{-}C^{-}D^{-} \) alleles. This frequency is intermediate to that found in the \( r^{LE} \) and \( r^{LI} \) series, and it differs from both at the 0.10 level of significance, but not at the 0.05 level (contingency \( \chi^2 = 2.82, p = 0.093, \) against the \( r^{LE} \) series and \( 3.22, p = 0.073, \) against the \( r^{LI} \) series).

Enzyme activities in mutant adults: Male flies were used for all enzyme determinations because females normally contain significant amounts of oocytes and retained embryos, and it has been shown that pyrimidine pathway enzyme activities are high in eggs and embryos (Brothers et al. 1978; Mehl and Jarry 1978; Rawls 1979a). Therefore, apparent enzyme levels exhibited by whole female flies are poor indices of \( r \) expression, since activity differences shown by mutant females might reflect fecundity differences rather than genuine defects in enzyme protein(s).

A number of new \( r \) mutants were assayed for CPSase, ATCase and DHOase activities, and data from these experiments are presented in Table 2. For each mutant, two separate samples of flies were homogenized and assayed. Although some variance is apparent between these replicate determinations, these data permit certain conclusions regarding the correlations between the complementation behaviors of \( r \) mutants and the enzyme defects exhibited by those mutants. For example, it appears that negative complementation accurately predicts specific enzyme deficiencies. Mutants that are \( A^{-} \) by complementation criteria are uniformly deficient in ATCase activity (i.e., they display 25% or less of \( r^{L+L} \) activity); mutants that are \( C^{-} \) are deficient in CPSase; \( D^{-} \) mutants are deficient in DHOase activity. Since our mutant collection lacks \( A^{+}B^{-}C^{-}D^{+} \) alleles, the data in Table 2 do not implicate the B function with a particular enzyme activity. Mutants that show partial complementation mostly support these correlations; that is, \( C^{+} \) mutants usually show low CPSase. The mutant \( r^{LE18} \) is a possible exception, but it should be recalled that \( r^{LE18} \) is a phenotypically “leaky” allele (Figure 3) and it does appear that CPSase is more reduced in this mutant than are ATCase and DHOase (Table 2).

A second conclusion drawn from the data in Table 2 is that positive complementation usually, but not always, predicts appreciably high enzyme activity levels. Exceptional cases are a few mutants that showed severely reduced levels of an enzyme activity for which their complementation behavior indicated normal levels. Most mutants that showed this “unpredicted” enzyme phenotype belong to complementation groups that show partial complementation for one \( r \) function and total noncomplementation for another \( r \) function. For example, the
The r locus in Drosophila

Table 2

Enzyme activities among mutant adult males

<table>
<thead>
<tr>
<th>Complementation group</th>
<th>r allele</th>
<th>CPSase</th>
<th>ATCase</th>
<th>DHOase</th>
</tr>
</thead>
<tbody>
<tr>
<td>A+B+C+D+</td>
<td>L+I</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(62±20)†</td>
<td>(318±805)†</td>
<td>(679±139)†</td>
</tr>
<tr>
<td>A-B+C+D+</td>
<td>LE11</td>
<td>63±1</td>
<td>0±7</td>
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</tr>
<tr>
<td>A+B+C±D+</td>
<td>LE12</td>
<td>5±8</td>
<td>10±12</td>
<td>4±1</td>
</tr>
<tr>
<td>A+B+C±D+</td>
<td>LE10</td>
<td>20±2</td>
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<td>39±9</td>
</tr>
<tr>
<td></td>
<td>LE18</td>
<td>36±3</td>
<td>71±8</td>
<td>91±9</td>
</tr>
<tr>
<td>A+B+C-D+</td>
<td>LE2</td>
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<td>139±18</td>
<td>67±23</td>
</tr>
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<tr>
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</tr>
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<td>LX5</td>
<td>13±6</td>
<td>9±6</td>
<td>0±1</td>
</tr>
</tbody>
</table>

* For each mutant genotype, two samples of flies were prepared and assayed independently, alongside samples of rL+I flies. The enzyme activity values obtained from each mutant sample were normalized using the values obtained from the parallel rL+I sample, which is defined as 100%. Data are presented as the mean of the two independent sample values ± the variation of the independent sample values from the mean.

† Values in parentheses are the mean rL+I enzyme specific-activity values observed in eleven experiments, expressed as mean picomoles of product formed per min per mg protein ± the standard error of the mean.

Complementation behaviors of the A+B±C−D+ allele rLE12 and the A+B+C±D− alleles, rLE13 and rLX5 might predict that rLE12 has normal ATCase and DHOase and that rLE13 and rLX5 have normal ATCase. However, each of these mutants exhibited severe reductions in all three enzyme activities (Table 2). The only other mutant showing an enzyme level that was not predicted by its complementation behavior was rLE1, an A+B+C+D− allele that showed reduced CPSase.
Otherwise, the enzyme activity levels exhibited by the great majority of mutants support the simple direct relationships of complementation unit A function with ATCase activity, C function with CPSase activity and D function with DHOase activity.

**DISCUSSION**

The r locus is a relatively large genetic region, based upon its recombination map length (Fristrom and Yund 1973), and the high frequency with which r mutants arose in the experiments reported here corroborates the recombination evidence. However, for at least three reasons, we believe that the mutation frequencies presented in Table 1 are underestimates. (1) The screening protocol employed in these experiments detects only mutations that are sufficiently impaired in r function to express the r wing phenotype, and we undoubtedly missed a large number of cryptic, subliminal mutations such as those described by Falk and Nash (1974). (2) Mutation mosaicism affects the detection of r mutants in these screens, since many and probably most mutations induced with EMS and ICR-170 in Drosophila are mosaically expressed in first-generation flies (Lee 1976). Although r is expressed autonomously in mutant tissues (Green 1963; Fausto-Sterling 1971), the detection of a mosaic r fly depends upon the inclusion of wing tissue within the mutant tissue sector of the fly. It is likely that many new mutations were not detected in these screens due to the new allele being present in only nonwing tissue of first-generation flies. (3) Up to half of all of the induced complementing mutants were not detected, since any new complementing allele would have an equal likelihood of arising within a female heterozygous for either r9 or r71b26 (Figure 2). In combination with one of these two alleles, most new complementing alleles would produce a normal-wing fly and the new mutation would therefore go undetected.

These considerations suggest that the actual frequencies of r mutations, complementing mutants in particular, are underrepresented by the data in Table 1, but they do not necessarily suggest that the screens were incapable of detecting most types of r mutants. Indeed, aside from leaky, normal-wing mutants, we doubt that any significant class of common r mutations could have systematically escaped detection. For example, the screening protocol should have permitted the detection and isolation of r mutants that are recessively lethal under standard culture conditions, so long as that lethality was complemented by r9 or r71b26, and we methodically sought lethal r mutants in these experiments. Although new r mutations were often recovered in chromosomes that were inviable in males, in all cases but one the lethality proved to be due to a second-site mutation that complemented Df(1)rD17. The sole exception was a presumed large chromosome deficiency. Therefore, while many r mutants are pyrimidine auxotrophs (Norbly 1970; Falk and Nash 1974), we have found no evidence that r function is essential for survival under standard culture conditions in yeast-based medium.

The A−B+C−D+ complementation group is represented by a single rLE mutant, and we recovered no new A+B−C−D+ alleles (Figure 4). These types of
mutants were also uncommon among mutants collected by previous investigators (FAHMY and FAHMY 1959; GREEN 1963). It should also be pointed out that another rare type of $r$ mutant, described as complementation group IV-V by CARLSON (1971), is not included among the new mutants described here. Perhaps this extinct type of allele would complement both $r^g$ and $r^{71238}$ and would have escaped detection in our screens.

Based on the genetic studies of CARLSON (1971) and enzyme activity measurements using certain mutants described by CARLSON, a variety of investigators have concluded that certain complementation units within the $r$ locus control each of the enzyme activities CPSase, ATCase and DHOase (Norby 1973; JARRY and Falk 1974; Rawls and Fristrom 1975; Fausto-Sterling 1977). Our results are in general agreement with these previous findings and show that what we call the A complementation unit controls ATCase activity, C controls CPSase activity and D controls DHOase activity. This correlation of the genetic complementation map and enzyme activity defects, together with the known co-linearity of the genetic fine-structure and complementation maps (Green 1963; CARLSON 1971), implies that each enzyme activity within the CPSase-ATCase-DHOase complex in Drosophila is encoded by a distinct region of the $r$ locus. It also follows that each enzyme activity probably resides within a distinct region of the trienzyme complex. For example, if $r$ encodes a single protein, the genetic and enzyme data suggest that each enzymatic function is performed by a unique domain within the trienzyme protein.

Although most of the mutants examined displayed agreement between their complementation behavior and their enzymatic phenotypes, a few mutants showed reduced levels of enzymes that were not predicted by their complementation behaviors (Table 2). Three of these exceptional mutants belong to the unusual complementation groups $A+B^-C^+D^-$ and $A+B^-C^+D^-$ and the other belongs to the more common $A+B^-C^+D^-$ group. A variety of explanations might account for the characteristics of these mutants: transcriptional and translation polarity effects, conformational interactions within the CPSase-ATCase-DHOase complex, and differential degradation of enzymatic domains within the complex. The available evidence does not allow us to distinguish among these diverse possibilities. Furthermore, it cannot be assumed that enzyme levels observed in adult flies should be directly related to genetic complementation behavior. For example, mutations within the $r$ locus that affect the susceptibility of the trienzyme complex to proteolysis might be expressed quite differently in diverse tissues and at different periods of the life cycle. Phenocritical periods for $r^+$ expression are oogenesis, embryogenesis and the pupal period (CARLSON 1970), and it is probable that genetic complementation behaviors of $r$ mutants are dictated by enzyme levels during those periods within tissues such as primordial wing and ovary. Therefore, adult enzyme activity levels might be misleading, and restraint must be practiced in interpreting their genetic significance.

The most striking conclusion from these studies is that different mutagens produce different classes of $r$ mutations, and this finding is undoubtedly related to the functional organization of the $r$ locus, as well as to the mutational speci-
ficities of EMS, ICR–170 and X rays. For example, most rLE mutants (19 of 25) are complementing alleles (Figure 4). The actual frequency with which EMS induces this type of mutant is certainly higher, since the screen protocol we used fails to detect up to one-half of all complementing mutants. Therefore, a theoretical estimate for complementing alleles among the rLE collection should double the number observed, giving a frequency estimate of 86% (38 of 44 potential mutants). Other studies found a preponderance of complementing alleles among EMS-induced, visible r mutants (Falk and Nash 1974; Mohler 1977). The observations that some EMS-induced r alleles are phenotypically leaky (Figure 3; Falk and Nash 1974) suggest that EMS produces missense mutations, which agrees with the popular conception of EMS mutagenesis. When fed to adult males, EMS participates in sperm DNA ethylation (Lee 1975), and DNA ethylation is presumed to lead to nucleotide transitions in bacteriophage (Lawley and Brookes 1963; Krieg 1963) and in yeasts (Prakash and Sherman 1973). EMS-induced mutations in Drosophila are mostly simple, single-site mutations (Suzuki et al. 1967; Jenkins 1967; Lim and Snyder 1974). At the Adh and ry loci, EMS-induced mutants appear to affect the primary structures of the respective protein products of these genes, alcohol dehydrogenase and xanthine dehydrogenase (Vigue and Sofer 1975; Schwartz and Sofer 1976; Gelbart, McCarron and Chovnick 1976). Therefore, EMS probably produces mostly nucleotide substitution mutations in Drosophila, and these should include nonsense as well as missense events.

In contrast to the rLE mutant series, all of the rLI mutants are phenotypically strong mutants (Figure 3), and all but one of these are A–B–C–D– alleles (Figure 4). These characteristics of rLI mutants agree with the presumed specificity of ICR–170 mutagenesis to produce frameshift mutations. ICR–170 induces mostly frameshifts and related microlesions in bacteria (Ames and Whitfield 1966; Creech et al. 1972) and in fungi (Malling 1967; Brusick 1969; Culbertson et al. 1977). In Drosophila, mutations at the dumpy locus induced with simple alkylating agents and X rays include a variety of recessive viable, as well as lethal, mutations, but apparently all ICR–170-induced dumpy mutants are recessive lethals (Carlson and Oster 1962; Carlson, Sederoff and Cogan 1967; Jenkins 1967; Grace 1970). Although no rigorous proof of frameshift mutations exists in the Drosophila literature, given the evidence in microorganisms and the characteristics of rLI mutants, it seems reasonable to suspect that the great majority of rLI mutants result from frameshifts and related microlesion mutations.

At least a few of the rLI mutants might be other than frameshift mutations. Until cytogenetic and mapping experiments are carried out on all of these mutants, it remains possible that chromosome rearrangements are involved in some of the rLI mutants. However, the fact that all rLI mutants obtained are hemizygous viable argues that most do not involve such rearrangements, and evidence has been presented previously that ICR–170 does not normally produce such events (Carlson and Oster 1962). ICR–170 mutagenesis is known to produce some nucleotide substitutions in yeast (Culbertson et al. 1977), and it is pos-
sible that the in vivo degradation of ICR-170 might produce a simple, alkylating nitrogen mustard that causes missense-like mutations in Neurospora (Ong 1970). Woodward and Gander (1974) found that ICR-170 induces temperature-sensitive lethal mutations in Drosophila and suggested that these might be missense mutations. One $r$ mutant of particular interest, the $A^+B^+C^+D^-$ allele $r^{Li8}$, could be an ICR-170-induced missense mutant. On the other hand, however, if $r$ is transcribed and translated via a polycistronic mRNA, a frameshift mutation within the translationally distal region of the locus could exhibit the $A^+B^+C^+D^-$ pattern; this is an alternate explanation for $r^{Li8}$. Additional experiments are in progress toward better genetic and molecular definition of this mutant.

The $r^{Lx}$ mutant series exhibits properties intermediate between the $r^{Le}$ and $r^{Li}$ series. Almost one-half (five of 12) of the $r^{Lx}$ mutants are complementing alleles (Figure 4). For four of these complementing alleles (e.g., $r^{Lx1}$, $r^{Lx2}$, $r^{Lx4}$ and $r^{Lx12}$), the likelihood of their detection in our screens was 50%, so that we may assume that approximately four comparable $r^{Lx}$ mutants were induced but not detected. A theoretical estimate for the frequency of complementing $r^{Lx}$ mutants should include these four presumed, undetected alleles, as well as the putative deficiency that was inferred to be a noncomplementing $r$ mutant from incomplete complementation data. The theoretical estimate for complementing alleles among the potential, visible $r^{Lx}$ mutants is therefore 53% (nine of 17). This estimate agrees well with previous studies in which about one-half of X-ray-induced $r$ mutants were complementing alleles (Fahmy and Fahmy 1959; Green 1963). The $r^{Lx}$ mutants probably represent a variety of mutational events. Ionizing radiation has long been known to induce chromosome breakage and rearrangements in Drosophila (reviewed by Sankaranarayanan and Sobels 1976). We apparently recovered one large deficiency and until the appropriate cytogenetic and fine-structure mapping studies are completed, we cannot say whether any of the other $r^{Lx}$ mutants are associated with chromosome rearrangements. However, it is likely that most of these mutants are simple mutations. Numerous X-ray-induced mutations at a variety of loci in Drosophila behave as simple, single-site mutations (Green 1963; Chovnick et al. 1969; Gelbart et al. 1974), and X-rays are known to produce simple mutations in fungi, including apparent nucleotide substitutions and frameshifts (Malling and Deserres 1973).

The distinctive properties of the $r^{Le}$, $r^{Li}$ and $r^{Lx}$ mutant series probably reflect the transcriptional and translation organization of the $r$ locus, and aspects of this organization are suggested by recent studies showing that CPSase, ATCase and DHOase exist in a trienzyme complex in animal cells. The most detailed studies on this complex have been performed with the mammalian complex, which is a homomultimeric protein within which each monomer possesses all three enzyme activities. The monomer migrates as a single protein band on electrophoresis under strong denaturing conditions (Mori and Tatibana 1975; Coleman, Suttle and Stark 1977), but attempts to identify and quantify the polypeptide ends of this protein were unsuccessful (Coleman, Suttle
When obtained from Drosophila embryos or cultured cells, CPSase, ATCase and DHOase co-sediment through sucrose gradients, extensively co-purify, and are all inhibited by an antiserum directed against purified ATCase (Brothers et al. 1978; Jarry 1978). Jarry (1978) has presented evidence that under certain conditions this complex contains a variety of polypeptides and that these polypeptides probably result from limited proteolysis of a large trienzyme polypeptide. The simplest interpretation of these findings is that the CPSase-ATCase-DHOase complex monomer consists of a single, unbranched polypeptide chain, as is the case for a number of other multienzyme complexes in eukaryotes (see review by Giles 1978). However, these findings are also consistent with an alternative explanation that the monomer is composed of multiple polypeptide chains that are covalently cross-linked such that they are inseparable by electrophoresis under denaturing conditions. Based upon the current information, three conceivable models might explain the synthesis of the CPSase-ATCase-DHOase complex monomer, and these models pose quite different mechanisms for \( r \) transcription:

Model 1: Several mRNA molecules each provide a polypeptide chain and these are associated and covalently cross-linked,

Model 2: A single mRNA is translated to yield successively several polypeptide chains, and these are associated and covalently cross-linked,

Model 3: A single mRNA is translated to produce a single, unbranched polypeptide chain.

Each of these models agrees with the behaviors of complementing \( r \) mutants, since these mutants show only that each enzymatic function is encoded by a distinct region of the \( r \) locus and that each enzymatic function is performed by a distinct domain within the CPSase-ATCase-DHOase monomer. However, noncomplementing \( r \) mutants constitute a group of diverse and potentially crucial mutants. Among these might be polarity mutants resulting from mutations within a cis-acting regulatory region, or frameshift and nonsense mutations within the polypeptide-encoding portion(s) of the locus. Frameshift and nonsense mutations would be expected to give polarity effects if \( r \) encodes a single, polycistronic mRNA. Finally, noncomplementing \( r \) mutants might comprise a variety of mutations, including missense mutations that result in conformational changes within the complex that effectively inactivate the entire complex. For any individual noncomplementing \( r \) mutant, distinguishing among these possibilities must await further genetic and molecular analysis of that particular mutant. As a group, however, the \( r^{L1} \) mutants are significant since it is likely that ICR-170 produces mostly microlesion mutations, including frameshifts, and since the overwhelming preponderance of \( r^{L1} \) mutants are strong, noncomplementing mutants. Although the frameshift nature of these mutants remains to be proven, we feel that the available evidence strongly indicates that frameshifts within the \( r \) locus normally produce severe polarity effects, as is expected if the CPSase-ATCase-DHOase complex monomer is translated from a single mRNA. Therefore, these data seem to contradict Model 1 above, but they do not distinguish between Models 2 and 3. Although Model 3 is the simplest inter-
pretation of the current biochemical evidence, additional genetic and biochemical studies are being pursued to test all possibilities.

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