I  Imidazole Compounds Accumulated by Purine Mutants of Neurospora crassa

II  Complementation Studies with Isoleucine-Valine Mutants of Neurospora crassa

Thesis by

Harris Bernstein

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ABSTRACT

Part I

Imidazole Compounds Accumulated by Purine Mutants of Neurospora crassa

The mycelia of mutants defective at seven adenine loci were investigated for imidazole accumulation. By the use of a paper chromatographic technique five imidazoles not present in wild type were discovered in the mutants. Two of these were isolated and characterized as the ribotide and riboside of 5-amino-4-imidazole-N-succinocarboxamide. The third compound was identified as 5-aminoimidazole riboside, and the fourth as 5-amino-4-imidazolecarboxamide riboside. The distribution of these accumulated compounds among the mutants allowed a correlation between the adenine loci and the steps of purine biosynthesis.
ABSTRACT

Part II

Complementation Studies with Isoleucine-Valine Mutants of *Neurospora crassa*

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 9 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 4 and 6 days after inoculation on minimal medium. Evidence was also presented to indicate that iv-3 mutants were blocked in the condensing step of isoleucine-valine biosynthesis. An extensive program of complementation testing was performed among mutants within both the iv-2 and the 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 could not be reconciled with any linear pattern. An interesting feature of the iv-3 studies, and to some extent of the iv-2 studies, was the finding that among the mutants found the same
patterns of complementation interaction often reoccurred. 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. It was hoped that these genetic regularities would prove, in the course of further biochemical investigations, to relate in a direct way to the structural properties of the enzyme molecule.
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<td>Bibliography</td>
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</tbody>
</table>
PART I
I. INTRODUCTION

When this work was initiated the pathway of purine biosynthesis had already been elucidated, through investigations with avian liver enzyme systems. The five steps prior to inosine-5'-monophosphate (IMP) formation had been found to involve precursors which were imidazole ribotides.

Also, at this time, genetic studies with a number of purine mutants of Neurospora crassa had resulted in the definition of eight adenine loci. A paper chromatographic procedure for the detection of imidazoles made it possible to study these compounds when accumulated in the mycelia of purine mutants. It was hoped that, with the aid of this method, the five biosynthetic steps leading to IMP formation could be correlated with their controlling genes.
II. THE BIOSYNTHESIS OF PURINE RIBOTIDES

Buchanan and his collaborators at the Massachusetts Institute of Technology have been able to formulate the complete pathway of purine biosynthesis (1). For the purpose of the present study, however, it is necessary only to detail the reaction steps following imidazole ring closure. These are given in Fig. 1.
Fig. 1. Reactions of adenosine-5'-phosphate biosynthesis after imidazole ring formation.
III. PURINE MUTANTS IN MICROORGANISMS

Purine mutants are frequently found in those organisms where screening procedures for nutritional mutants are applicable. In the subsequent sections purine mutants of Neurospora crassa will be discussed. Here the purine mutant studies in other organisms will be summarized briefly.

The results of many of the following growth experiments are now explicable in terms of known biosynthetic pathways. Thus the growth effects of guanine and xanthine supplements arise because IMP, in addition to forming AMP (Fig. 1.), is also converted into xanthosine-5'-monophosphate which in turn is aminated to guanylic acid. Furthermore adenosine-5'-triphosphate (ATP) serves as a precursor of histidine, contributing the nitrogen-1 and carbon-2 of the purine ring. The effects of histidine supplement have been explained by Moyer and Magasanik (2) in terms of histidine repression of the ATP cleaving enzyme. Other compounds enhancing growth, such as glycine, hypoxanthine and various imidazoles, are reactants in the purine biosynthetic pathway.

Fries and collaborators have described a number of purine mutants of Ophiostoma multiannulatum. They especially studied the effect on growth of various supplements to minimal medium. One mutant was found which could use adenine only in the presence of added histidine and glycine (3). Another was described which would use either adenine, hypoxanthine, or 5-amino-4-imidazolecarboxamide (4). Three
mutants required adenine but not hypoxanthine or guanine (5). Nineteen required either adenine or hypoxanthine but not guanine (6). Four mutants had a specific guanine requirement.

There have been a number of reports on the nutritional requirements and accumulation products of *Escherichia coli* purine mutants. Xanthine, guanine, hypoxanthine, 5-amino-4-imidazolecarboxamide, 5-formamido-4-imidazolecarboxamide and vitamin B12 were used as nutritional supplements (7-13). In addition Gots has presented evidence for the accumulation of SAICAR (10) (see Fig. 1.), AICAR (7, 14), AIR (11) and CAIR (15) by various mutants. Particularly interesting was a mutant B-97 which was defective in the two deacetylation reactions of purine biosynthesis, the conversion of SAICAR to AICAR and the conversion of AMPS to AMP (16).

Purine mutants of *Salmonella typhimurium* fell into two main classes -- those which would grow on adenine supplemented media alone, and those which specifically required thiamine and/or pantothenic acid in addition to adenine (17). Recombination data from transduction experiments grouped the adenine mutants into three clusters and the adenine-thiamine mutants into three more. Further subdivisions were made on the basis of substrate requirements and accumulation of intermediates. In one subgroup all eight mutants accumulated in their media a yellow pigment which had an ultraviolet absorption spectrum with maximum at 305 m. Evidence for the accumulation of 4-amino-5-imidazolecarboxamide by one mutant and SAICAR by four others was reported (14).
Pomper (18) has investigated five adenine requiring mutants in *Saccharomyces cerevisiae*. Roman (19) has described purine mutants at seven loci in *Saccharomyces*. Mutants defective at two of these loci (ad-1 and ad-2) accumulated a red pigment in their media. Another mutant ad-3R required histidine as well as adenine supplement.

Magasanik and others (13, 20, 21) with the aid of purine mutants of *Aerobacter aerogenes* studied the reactions by which guanylic acid is synthesized from IMP.

Pritchard (22) by recombination analysis in *Aspergillus nidulans* has described the clustering of four mutational sites responsible for adenine requirements in a small (.3 map units) region of the genetic map. Mutants defective at four other adenine loci have also been described by Galef (23), and Köfer (24).

Purine mutants have also been found in *Coprinus fimenterius* (25), *Aerobacter melanogenum* (26), *Penicillium notatum* (27), and *Bacillus subtilis* (28).

It is noted that with respect to supplement specificity and accumulation products the mutants of *N. crassa*, to be described below, are quite similar to the mutants of these other microorganisms. In addition the available data from these various organisms are consistent with the pattern of biosynthesis described for avian liver (Fig. 1.). This suggests that elucidation of the genetic control of purine biosynthesis in *N. crassa* will provide information of general applicability.
IV. PURINE MUTANTS OF NEUROSPORA CROASSA

All adenine mutants found to date have been allocated to eight loci (29, 30) and these are summarized in Table 1.

Table 1. Summary of mutants assigned to the eight adenine loci

<table>
<thead>
<tr>
<th>Locus</th>
<th>Mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ad-1</strong></td>
<td>3254*</td>
</tr>
<tr>
<td><strong>ad-2</strong></td>
<td>70004, 27363, 20708</td>
</tr>
<tr>
<td><strong>ad-3</strong></td>
<td>35203, 38701, 39709, 45801, 68306, A, B</td>
</tr>
<tr>
<td><strong>ad-4</strong></td>
<td>44206, 44415, F</td>
</tr>
<tr>
<td><strong>ad-5</strong></td>
<td>71104, J</td>
</tr>
<tr>
<td><strong>ad-6</strong></td>
<td>38610</td>
</tr>
<tr>
<td><strong>ad-7</strong></td>
<td>44411</td>
</tr>
<tr>
<td><strong>ad-8</strong></td>
<td>E</td>
</tr>
</tbody>
</table>

* Mutants with numerical designations came from the early mutant searches of Beadle and Tatum (31).
† Letter designations were given by Giles to groups of mutants related by heterokaryon complementation tests.

The **ad-3** and **ad-5** loci are both in linkage group I but about 10 map units apart (32). Deserres (33) has reported that all **ad-3** mutants fell into two groups on the basis of heterokaryon complementation. The **ad-2** and **ad-4** loci are 1.3 to 3.4 map units apart in linkage group III.
(32, 34). \textit{ad-3} is in linkage group IV, \textit{ad-7} in linkage group V, and \textit{ad-1} in linkage group VI.

It was shown by Mitchell and Houlahan (32) that adenosine and adenosine-3' phosphate would replace adenine as a supplement for all adenine mutants tested. Hypoxanthine serves as a supplement in place of adenine for all adenine mutants except those at the \textit{ad-4} and \textit{ad-8} loci (30, 32). McElroy and Mitchell (35) demonstrated that on media supplemented with histidine or methionine, in addition to adenine, growth of 44205 (\textit{ad-4}), but not 25310, 70004 and 35203 was more abundant than on adenine supplemented media alone. AMP-S and its aglycone were reported to be ineffective in promoting the growth of any of the adenine mutants (36).

Whitfield (37) and Giles (38) have shown that \textit{ad-4} mutants accumulate AMP-S. \textit{Ad-3} mutants were reported to accumulate hypoxanthine (30, 30). Double mutants defective at both the \textit{ad-4} and \textit{ad-3} loci were also found to accumulate hypoxanthine, indicating that the \textit{ad-3} block precedes the \textit{ad-4} block. All \textit{ad-3} mutants accumulate a purple pigment in their media. Mutant 44205 (\textit{ad-4}) has also been reported (32) to accumulate a pigment under certain conditions.

\textit{F} mutants and 44205 were reported (30) to lack activity for a bifunctional enzyme catalyzing the deacetylation of \textit{SAICAR} and AMP-S to \textit{AICAR} and AMP respectively. T. French is reported (1) to have found in wild type \textit{H. messei} all the purine biosynthetic enzymes present in the avian liver system.
METHODS

Extraction of mycelia

Mutants were grown in carboys containing 10 liters of minimal medium (31) supplemented with limiting amounts of adenine sulfate (usually 30 μg/ml). After 3-4 days growth under forced aeration the mycelia were harvested by pouring the contents of the carboys through cheesecloth. The mycelial mats were placed in a Waring Blender and boiling water was added (about 1 liter per 100 gm. of wet mycelium). After disintegration the extracts were filtered through Whatman No. 1 filter paper in a Buchner Funnel. The filtrates were then lyophilized overnight leaving thick syrupy extracts in the flasks. Five to 10 ml. of water was sufficient to take up the extract from 100 gm. of mycelia. Residual material was separated by centrifugation and washed several times with 1 ml. portions of water which were added back to the soluble fraction. The solutions contained all the imidazoles. The residues were discarded.

Detection of accumulated imidazoles

The redissolved extracts were spotted directly, or with dilution, on Whatman No. 1 paper and resolved by ascending chromatography in a variety of solvents. Isopropanol, H₂O, and conc. NH₃OH in the ratios 70:20:10 and 70:40:10 were the most useful solvent systems. After drying, the chromatograms were sprayed very lightly with
diazosulfonic acid reagent (40), and again very lightly with 5% Na₂CO₃. By this procedure five distinct imidazole compounds, not present in wild type were detected in extracts from the mutant strains.

Isolation of Compounds I and II (Table 3.)

To 14 ml. of the water soluble extract of 135 gm. wet weight of mutant 44206 an equal volume of methanol was added. A precipitate was formed which was washed with 50% methanol. The soluble fractions containing virtually all of compounds I and II were combined to a total volume of 23.5 ml.

A Dowex exchange resin (1-X2, 200-400 mesh), from which the fines had been removed by repeated washings, was equilibrated with 2M formic acid. This was placed in a column 2.2 cm. in diameter to a volume of 170 cm³. Water at 3.5⁰ was circulated through a jacket about the column. The flow rate of solvent was kept at about 1 drop every 15 seconds by maintaining the system under controlled positive pressure. A mixing vessel of 125 ml. capacity was included in the system to allow gradient elution according to the procedure of Thompson (41). The column was first washed with distilled water and then the sample was added. For elution the series of solutions listed in Table 2. were used. Successive solutions were added only after the pH of eluted fractions did not change measurably. Making use of an automatic fraction collector, 5-10 ml. portions were collected.
Table 2. Chromatographic solutions

<table>
<thead>
<tr>
<th>No.</th>
<th>Solution</th>
<th>Molarity of NH₄⁺</th>
<th>Molarity of anion</th>
<th>Measured pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>distilled water</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>ammonium acetate buffer</td>
<td>.4</td>
<td>1.2</td>
<td>4.28</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>.4</td>
<td>1.0</td>
<td>4.40</td>
</tr>
<tr>
<td>4</td>
<td>ammonium formate buffer</td>
<td>.4</td>
<td>1.1</td>
<td>3.28</td>
</tr>
<tr>
<td>5</td>
<td>acetic acid</td>
<td>-</td>
<td>1.0</td>
<td>2.53</td>
</tr>
<tr>
<td>6</td>
<td>formic acid</td>
<td>-</td>
<td>1.0</td>
<td>1.37</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
<td>-</td>
<td>6.0</td>
<td>1.17</td>
</tr>
</tbody>
</table>

Compound II began to elute at pH 1.3 (Solution 7). It came off the column in a total volume of 273 ml. After an additional 136 ml. had passed through, compound I began to appear and it came through in the next 32 ml. Further treatment consisted of neutralization with NH₄OH, lyophilization, and removal by sublimation at 65°C in a vacuum oven of the ammonium formate. The resulting samples were then used for analysis.

Separation of Compound III and IV (Table 3.)

An extract of mutant 45601 containing both compounds III and IV was subjected to a chromatographic procedure similar to that described above. Compound III was eluted by water very close to the front, and compound IV came off soon after. Though separation was achieved, both fractions were impure. However they were satisfactory for chromatographic comparisons.
RESULTS

The color reactions and distribution among the mutants of the five detected imidazoles are detailed in Tables 3 and 4.

Table 3. Color reactions of accumulated compounds.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
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<tr>
<td>Color Development</td>
<td>Before $\text{Na}_2\text{CO}_3$</td>
<td>red - orange</td>
<td>bright orange</td>
<td>bright yellow</td>
<td>yellow</td>
</tr>
<tr>
<td></td>
<td>After $\text{Na}_2\text{CO}_3$</td>
<td>fades to grey</td>
<td>fades to grey</td>
<td>grey spots-fades</td>
<td>red-blue</td>
</tr>
<tr>
<td>Probable Identity</td>
<td>SAICAR (ribotide)</td>
<td>SAICAR (riboside)</td>
<td>AIR (riboside)</td>
<td>?</td>
<td>AICAR (riboside)</td>
</tr>
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Table 4. Distribution of accumulated imidazoles

<table>
<thead>
<tr>
<th>Locus</th>
<th>Mutants</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
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</thead>
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<tr>
<td>ad-1</td>
<td>3285</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>ad-2</td>
<td>70004</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>27663</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>20705</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>ad-3</td>
<td>35203</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>33701</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td></td>
<td>33709</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>45601</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ad-4</td>
<td>44206</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>ad-5</td>
<td>71104</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ad-6</td>
<td>28610</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ad-7</td>
<td>44411</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Characterization

Compound I at pH 7 had an ultraviolet absorption peak at 260 m\textmu. By the method of Mejbaum (42) it was shown to contain a pentose moiety. A sample of SAICAR generously provided by T. French proved to be identical to compound I, both in color development and chromatographic mobility. When compound I and the known SAICAR were hydrolysed by alkaline phosphatase each formed a product identical on chromatograms to compound II.

Compound II at pH 7 absorbed maximally at 268 m\textmu, and was also shown to contain a pentose moiety. Using the procedure of Allen (43) it proved to lack a phosphate group. On hydrolysis at 105\degree with conc. HCl, for 15 hr. in a sealed tube, compound II yielded aspartic acid and glycine as determined by the I-O chromatographic system of Hardy, Holland and Nayler (44). The presence of aspartic acid on hydrolysis, the shape of the ultraviolet absorption curve, and position of the maximum compared well with the properties reported for SAICAR (14). The phosphate determination and the data from hydrolysis with alkaline phosphatase further showed that compound II is the pentoside, probably riboside, corresponding to SAICAR.

Compound III formed an orange-red Bratton Marshall reaction product (45) which absorbed maximally at 500-502 m\textmu. No absorption maximum in the ultraviolet region was detected. That compound III failed to bind to an anion exchange resin argues against the presence of carboxyl or phosphate group.
The Bratton Marshall product of AIR is reported to be salmon-orange and absorb maximally at 500 μm (46). Also this compound is reported to have no ultraviolet absorption maximum above 210 μm. All the properties observed for compound III are consistent with its identity being AIR (riboside).

The Bratton Marshall reaction product of compound IV was orange-red and absorbed maximally at 534-538 μm. This corresponds to none of the maxima reported for the imidazoles involved in purine biosynthesis. However since the concentration of this compound appeared to decrease as more care was taken in preparation of extracts, it seems likely that compound IV is a reaction product of compound III.

Ames and Mitchell (40) have reported that of a list of 16 compounds tested by their diazotization procedure, only 5-amino-4-imidazolecarboxamide (AICA) gave a blue reaction product. Subsequently AICAR was observed by the author to give this blue color. Compound V gives a color reaction identical to that of the aglycone and AICAR. Chromatographic evidence suggested that compound V was either the aglycone or the riboside of AICAR.
V. DISCUSSION

Knowing the distribution and probable identity of the detected imidazole compounds one can correlate the adenine loci and the steps of purine biosynthesis (Fig. 2.)

Fig. 2. Positions in purine biosynthesis controlled by adenine loci as judged from imidazole accumulations by mutants.

\[
\begin{align*}
\text{ad-2, ad-3 & ad-7} & \quad \text{ad-3} \\
\text{control reactions} & \quad \text{AIR} \quad \text{C-AIR} \quad \text{SAICAR} \\
\text{prior to imidazole} & \quad \text{AMP} \quad \text{AMP-S} \quad \text{IMP} \quad \text{FAICAR} \quad \text{AICAR} \\
\text{ring closure} & \quad \text{ad-4} \quad \text{ad-1, ad-5}
\end{align*}
\]

* Giles et al. (30) have shown that mutants at the ad-4 locus are deficient in a deacylase which splits both SAICAR and AMP-S.

† ad-3 mutants were shown also by Giles et al. (30) to be blocked in the conversion of IMP to AMP-S.

Since ad-2, ad-3 and ad-7 mutants can use IMP or hypoxanthine in place of adenine as a growth supplement (47) and yet accumulate no imidazole compounds, they can be assigned to steps preceding imidazole ring closure.

This conclusion is in accord with an observation of Mitchell et al. (47). All ad-3 mutants accumulate a distinctive
purple pigment in their growth media. The purple pigment, which seems to have associated with it a 305 m\(\mu\) ultraviolet absorption maximum, is probably a reaction product of compound III. Double mutants or 35203 (\textit{ad-3}) in conjunction with \textit{ad-2}, \textit{ad-6} and \textit{ad-7} mutants, accumulate no purple pigment. The double mutant 35203, 44206 (\textit{ad-4}) does accumulate the pigment. This again places the \textit{ad-2}, \textit{ad-6} and \textit{ad-7} block prior, and the \textit{ad-4} block subsequent to the reaction controlled by \textit{ad-3}.

Colorless solutions of SAICAR (compound II) will turn red in time, especially at low pH or on exposure to air. The reddish appearance of 44206 (\textit{ad-4}) mycelia is probably due to this effect. The percentage yield of the ribotide and riboside of SAICAR in the dried mycelia of 44206 can be calculated from the observed optical densities at the ultraviolet absorption maxima and the extinction coefficient given by Lukens (43). The yields of the riboside and ribotide were found to be 2.1% and .023% respectively, of the dry weight of mycelia. The yield of AIR (riboside) in the mycelia of 45206 as judged by the intensity of the color reaction in crude extracts is probably as high as the yield of SAICAR (riboside) in 44206. It is suggested that these mutants might be useful for large scale isolation of these otherwise hard to prepare compounds.

The two intermediates C-AIR and FAICAR, known to be the least stable of the imidazoles, were not detected in any extracts.
BIBLIOGRAPHY


PART II
INTRODUCTION

Historical

When two mutant genomes each defective for a specific function are allowed to share a common cytoplasm, the functions are often completely or partially restored. This phenomenon is called complementation. In *Neurospora crassa* complementation occurs as the result of hyphal fusion and heterokaryon formation. The occurrence of fusion between fungal hyphae, of the same mating specificity, was observed as early as 1888 by Ward (1). Dodge (2) described the formation of heterokaryons between two strains of *Neurospora tetrasperma*, by the migration of nuclei across the openings at the points of anastomosis. Included in this study were two cases in which slow growing strains of the same mating type formed rapidly growing heterokaryons. These were probably the first recorded cases of heterokaryon complementation. Beadle and Coonradt (3), using *N. crassa*, performed the first complementation studies among mutants having specific nutritional requirements. In these studies two mutants were considered to complement each other if their paired inocula grew significantly on minimal medium. After producing heterokaryons, these investigators could recover the mutant components, among the ascospores produced in a cross of the heterokaryon to wild type. It was also in this report that the use of heterokaryon formation as a test for allelism was first suggested.
Mitchell, Pittenger and Mitchell (4), and Pittinger (5) described the formation, in crosses between two mutants, of ascospores which gave rise to mycelia heterokaryotic for both mutant types. Heterokaryons arising in this fashion have been termed "pseudo-wild" types, and although their occurrence has subsequently been used as a test for complementation, there is still no direct evidence as to how they are formed.

L. Garnjobst (6) discovered the existence of two loci which controlled heterokaryon compatibility i.e. the ability of two strains to form heterokaryons. In a similar study Holloway (7) demonstrated the presence of four or possibly five genes controlling heterokaryon compatibility. The possibility of a relationship to those genes described by Garnjobst was not investigated. By the application of micro-techniques it was shown, in this work, that incompatible strains did not even undergo hyphal fusion. These investigations made it apparent that, in order to obtain reliable complementation data, care must be taken to use mutants whose backgrounds are as isogenic as possible.

It has been found in subsequent studies that mutants defective in separate genes, as defined by biochemical and recombinational criteria, always complement if heterokaryon compatible. Mutants defective in the same gene have also been found to complement and this has been termed "interallelic complementation".
The first clear case of interallelic complementation in *A. oryzae* was reported by Mitchell and Mitchell (8). It was shown that the three known mutants defective at the pyrimidine-3 locus produced "pseudo-wild" type progeny in all possible pairwise crosses. Pyrimidine-independent heterokaryons could not be obtained by mixing conidia of the mutant strains. However, individual mutant conidia obtained from the pseudowild cultures could be used to reconstitute pyrimidine-independent heterokaryons.

Another early case of interallelic complementation reported by Mitchell (9) involved three mutants defective at a pyridoxine locus. Again, each of the possible pairwise crosses yielded "pseudowild" types, but pyridoxine-independent heterokaryons could not be formed by merely mixing the conidia of two strains. It was possibly significant that in both of these studies, the mutants used had been isolated about 10 years previously, and probably been subject to repeated subculture. This could allow considerable divergence with respect to compatibility alleles, and hence the apparent inability of mixed conidia to complement.

Recent studies

As the result of increased interest in the problem of interallelic complementation a number of investigations have recently been carried out. In those studies involving many mutants, the results of pairwise growth tests are scored in the form of a matrix of positive and negative responses.
Frequently found are mutants which do not complement with two or more mutants, which later however do complement with each other. These non-complementing mutants have been termed "overlap" mutants, and it is on the basis of these that complementation maps are constructed. All mutants having the same pattern of complementation are placed in one subgroup represented on the map by a line. Non-complementing subgroups are represented by overlapping lines, whereas complementing subgroups are not overlapping. This is illustrated in Fig. 3. Subgroup A does not complement with B and F, but these do complement with each other. Since, in all studies presented to date, the data have allowed the representation of every subgroup by a single uninterrupted line, complementation maps can thus far be said to possess the property of linearity.

Catcheside and Overton (10) have described the complementation of 40 ultraviolet irradiation-induced mutants defective at the arginine-1 locus. Thirty of these mutants would not complement with any of the others, and were drawn as "complete overlaps" on the complementation map. The remaining 10 mutants were placed in 5 additional subgroups. The linear complementation map presented was considered to be a completely consistent representation of the data.

Eleven mutants defective at the locus controlling glutamic acid dehydrogenase formation were studied by Fincham (11). Also included in this study was a strain producing an altered form of this enzyme, which had arisen by apparent back-mutation of one of the eleven primary mutants. The complementation map
arrived at contained 6 subgroups. There was only one "complete overlap" mutant. In a further study (12), Fincham found that the glutamic acid dehydrogenase activity, arising from complementation, in two cases, resided in a protein with physical properties different from those of the wild-type enzyme.

Woodward, Partridge and Giles (13), and Giles (14) have presented a complementation map of the adenine-4 locus, containing 72 "complete overlap" plus 51 complementing mutants. However, 44 of the 51 complementing mutants had arisen secondarily by irradiation of revertants or partial revertants, of two of the primary mutants. All mutants either arose spontaneously, or following induction by X-Ray or ultraviolet irradiation. The linear complementation map presented had 17 complementing subgroups. It was also shown (15) that this complementation involved a restoration of the adenylosuccinase activity, which is absent in all of these mutants. Further in vitro experiments by Woodward (16) suggested that this restoration of activity resulted from protein-protein interaction. Woodward also reported that the strength of complementation between two mutants, as measured by growth and adenylosuccinase activity, was weakest for those adjacent on the complementation map, and became stronger as the mutants were more separated. The most distant mutants gave no more than 2% of wild-type enzyme activity.

A study was made by Case and Giles (17) of mutants defective at the pantothenic acid-2 locus, which controls the conversion
of keto-valine to keto-pantoic acid. Of the 75 mutants obtained, 3 had arisen spontaneously, 52 were X-Ray induced and 17 were ultraviolet light induced. Fifty-two of the total were represented as "complete overlap". The remaining 23 were allocated to 12 subgroups from which was formulated a linear complementation map. On the basis of prototroph frequencies, obtained in mutant by mutant crosses, it was also possible to order the mutational sites in a linear array. Most of the prototrophs in these crosses can be expected to have arisen by the non-reciprocal type of recombination known as "gene conversion". Further checking of this linear order was carried out using the prototroph frequencies obtained from crosses of double mutants (both sites within the pan-2 locus) to single pan-2 mutants. The order of mutants thus achieved showed a striking correlation to the order of mutants on the complementation map. However, exceptions to this co-linearity were clearly demonstrated. The exceptions can be described pictorially by saying that two mutant sites allocated to the right side of the genetic map, had to be placed near the center of the complementation map.

The results of two further inter-allelic complementation studies have thus far only been reported briefly in the form of abstracts. Ishikawa (18) presented work with 242 primary and 64 secondary mutants defective at the ad-3 locus, which controls adenylosuccinic acid synthetase activity. The most interesting aspect of this study was the finding of a definite parallel between the genetic and complementation maps, with
several exceptions. Woodward and Cook (13) working with mutants defective at the pyrimidine-3 locus also reported discrepancies between the genetic and complementation maps.

It was reported in studies of both the arginine-1 (10) and adenine-1 (12) loci that many of the "overlap" mutants used were revertible to independence from nutritional requirement. In addition, the recombination experiments of Case and Giles (17) demonstrated that "overlap" mutants behaved as if, in each case, only a small site was defective. These findings strongly suggest that "overlap" mutants cannot be regarded as genetic deletions.

Wagner, Somers and Bergquist (20) have described complementation studies with mutants, requiring isoleucine and valine, similar to those used by the present author. A comparison of the results of these two studies is therefore relegated to the Discussion.

Significance

The motivation for carrying out these complementation studies was the expectation that they would be helpful in elucidating some of the finer details of gene function. Therefore, it would be well to discuss, in a general way, the other methods employed in the study of this problem, and how their findings are related to complementation phenomena. The two operationally independent lines of experimentation that have been most useful in characterizing the relationship of gene structure to function are: (1) analysis of metabolic and structural defects in mutants by the use of biochemical
methods, and (2) recombination analysis.

The study of accumulation products and supplementation experiments, in addition to other methods have usually allowed the arrangement of nutritional mutants into discrete groups, each representing a specific metabolic defect (21). Further analysis has generally yielded the result that the mutants in each group were defective for a specific enzymatic activity (22). Genetically caused defects in presumably non-enzymatic proteins, such as human hemoglobin (23) and bacteriophage head protein (24), have also been reported. Studies of 8 different human hemoglobin types by even more refined approaches suggest that these defects can be pinpointed to specific amino acid alterations in the peptide chains of the protein molecule (23).

Using recombination data for the construction of linear genetic maps, it has been shown that mutants concerned with the same biochemical reaction step tend to be clustered in a small region of the genetic map (21). It is because of this correlation that the gene is considered to be a discrete linear segment of genetic material with a single function.

The mechanism of physical exchange involving the reciprocal breakage and fusion of homologous chromosomes, that has generally been envisioned to account for the recombination of distant mutational sites, does not seem applicable to the recombination of mutants defective in the same gene. Recombination between two distant sites is
characterized by the reciprocal formation, during a single meiosis, of both possible recombinant progeny. However, there is substantial evidence (25, 26 and 27) to indicate that interallelic recombination involves a non-reciprocal formation of recombinants. It is hard to imagine how a mechanism of physical exchange could allow the formation of reciprocal products at one level and non-reciprocal products at another level, if all linked mutational sites are arranged in a one dimensional array. At present there is no completely adequate explanation for these findings.

It is significant that all mutants judged to be defective in a single gene by the biochemical and recombination criteria can also be unambiguously allocated to that gene by complementation, i.e. all mutants defective in the same gene do not complement with the "complete overlap" mutants for that gene but do with all mutants representing other genes. Also, it appears that interallelic complementation results from the direct interaction of gene products (16), and that the complementation maps reflect this interaction. Therefore it seems likely that the studies of interallelic complementation can yield genetic information at a level of refinement exceeded only by the most advanced biochemical and recombinational approaches. It is possible that a direct correlation will be found between the complementation subgroup and a sub-structure of the protein molecule.

The present work includes an intragenic complementation study at each of two loci concerned with isoleucine and 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 (29). The strain used in all induction experiments was inos. 29501a which originated in a sample of nitrogen mustard treated spores descended from the standard wild cross Em 5256A X Em 5297a. Conidia were harvested from cultures grown at 35°C on 20 ml. of the complete medium described by Horowitz (29).

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 x 10⁷ conidia per ml. All chemical treatments were terminated by dilution into cold phosphate buffer (1/15 M, pH 7.0). In all experiments, except 1, 2 and 3, the recommended shaking step (29) 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 (30) to which was added agar 6.5 mg./ml., glucose 2.0 mg./ml., glycerol 2.0 mg./ml., sorbose 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.
<table>
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**Table 1**

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<td>No</td>
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<td>Yes</td>
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</tbody>
</table>

**References**

- Abrevá, E.
- García, J.
- Martínez, A.
- Ortega, M.
- Pérez, L.
Complementation tests

Complementation tests were regularly performed in large petri dishes (radius 7 cm.) containing 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 x 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°C and growth was recorded daily. Overgrowth of spots by neighboring, early-positive tests usually limited the usefulness of plates to from 6 to 10 days.

Another, more sensitive, method was also employed for complementation testing. Two inch test tubes containing 1 ml. each 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 of these were added inocula from two mutants. After 3 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 twenty 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 minimal medium (35) supplemented with L-isoleucine 100 μg./ml., L-valine 100 μg./ml. and inositol 10 μg./ml. in addition to 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 80°, the plates were placed at 36° 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 thirteenth mutant found in series 18. The mutants of Experiment I were unique in that they were obtained from plates overlayed with minimal medium containing 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 (28 and 31). 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.
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</table>

Table 2

Trends and characteristics of substances
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 \textit{iv-1*}, \textit{iv-2*}, \textit{iv-3}, \textit{val-1} and \textit{val-2}. All tests involving two mutants from different groups were always positive within a day after inoculation. The data from all inter-group 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 \textit{val-1} or \textit{val-2} group. Mutants in the \textit{iv-1} group were all observed to grow on minimal medium between 4 and 5 days. For the other groups, the proportion of mutants displaying growth on minimal medium before 5 days was not more than a few percent of the total number. Among the 1,012 \textit{iv-1 x 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 designation \textit{iv-1} was first used by Barratt et al. (37) as an assignment for mutant 16117 and its possible alleles. The designation \textit{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 \textit{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.
The complementation behavior of isoleucine-valine mutant 16117, obtained by Beadle and Tatum (36), 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 (33) that 16117 accumulates the intermediates $\alpha,\beta$-dihydroxyisovaleric acid and $\alpha,\beta$-dihydroxy-$\beta$-methylvaleric acid (Fig. 1.), and in addition lacks the dehydrase which converts these compounds to $\alpha$-ketoisovaleric acid and $\alpha$-keto-$\beta$-methylvaleric acid respectively (33). 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.
Fig. 1 Biosynthesis of Isoleucine and Valine

COOH
C=O \rightarrow \text{HO-C-CH}_3
CH_3

\text{PYRUVIC ACID} \quad \text{\(\alpha\)-ACETOACID}

\begin{align*}
&\text{\(\alpha\)-ACETYLACID} \\
&\text{\(\alpha\)-HYDROXY-ISOVALERIC ACID} \\
&\text{\(\alpha\)-KETOISOVALERIC ACID}
\end{align*}

\begin{align*}
&\text{+} \\
&\text{CONDENSING ENZYM} \\
&\text{REDUCTO-ISOERASE} \\
&\text{DEHYDRASE} \\
&\text{TRANSAMINASE}
\end{align*}

\text{COOH}
C=O \rightarrow \text{HO-C-CH}_2-CH_3
CH_3

\text{PYRUVIC ACID}

\begin{align*}
&\text{\(\alpha\)-KETO-}
&\text{BUTYRIC ACID} \\
&\text{\(\alpha\)-ACETO-}
&\text{BUTYRIC ACID} \\
&\text{\(\alpha\)-HYDROXY-}
&\text{\(\beta\)-METHYLVALERIC ACID} \\
&\text{\(\alpha\)-KETO-\(\beta\)-METHYL-
&\text{ACID}
\end{align*}

\text{COOH}
C=O \rightarrow \text{HO-C-CH}_2-CH_3
CH_3

\text{\(\alpha\)-ACETOACID} \\
\text{\(\alpha\)-HYDROXY-ACID} \\
\text{\(\beta\)-METHYLVALERIC ACID} \\
\text{\(\alpha\)-KETO-\(\beta\)-METHYL-
\text{ACID}
Table 3

Intergroup complementation tests

<table>
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<th>Groups</th>
<th>Number of tests performed (all positive)</th>
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<td>iv-1 x iv-3</td>
<td>355</td>
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<td>iv-1 x val-1</td>
<td>77</td>
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<tr>
<td>iv-1 x val-2</td>
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<td>val-1 x val-2</td>
<td>18</td>
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The iv-2 group

The 94 mutants allocated to the iv-2 group were tested in 332 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 (Fig. 2.). 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 21-5 and 20-5 could have been reversed. Almost all positive responses were initiated between 3 and 5 days after inoculation. There was some tendency for heterokaryotic growth to be more vigorous as the "distance" on the map, between the mutants involved, 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" 21-5 and 20-5, complementation was not observed until 7 and 8 days, respectively.

The 76 completely overlapping mutants had the following mutagenic origins: 42, hydrogen peroxide with formaldehyde; 23, ultraviolet light; 7, nitrogen mustard; 3, X-Rays; and 1, bromodeoxyuridine. Of particular interest were 5 mutants, representing 3 different mutagenic origins, which displayed identical complementation patterns (Fig. 2. second subgroup
### Table 1

<table>
<thead>
<tr>
<th>MM</th>
<th>L0</th>
<th>22-15</th>
<th>22-8</th>
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### Table 2

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<td>10-8</td>
<td>8-6</td>
<td>2-15</td>
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More notes on complete overlap and the Zr-Zr group.
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 Fig. 3, was formulated. The complementation behavior of 428 of the mutants was completely consistent with this linear representation, but the remaining 4 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 6 patterns of complementation. However, 326 of these were 0 mutants derived from U.V. treatment. This 6 subgroup representation was initially observed early in the testing program when only U.V. induced mutants were available. Of the 52 non-U.V. mutants subsequently obtained (Table 4) 25 did not complement and were placed in subgroup A, 23 could be placed in one of the other 5 established subgroups, and 4 gave exceptional results.

The number of possible pairwise tests \( T \), where \( n \) is the number of mutants tested, is \( n \left( \frac{n-1}{2} \right) \). When \( n \) is 432,
Fig. 3. Complementation map of the iv-3 group

A (50)**

B (28)

C (333)

D (5)

E (5)

F (7)

(PLUS 4 EXCEPTIONAL MUTANTS)**

* 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.
### Table 4

Distribution of mutants found in successive selection experiments

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**Total**

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* Several mutants, which grew on minimal medium to the extent that classification was interfered with, were not included.
T is 93,096. The number of tests actually performed, 10,500, was only 11% 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 x C tests were actually performed (all giving negative results), it seemed unnecessary to prolong this phase of the testing program. The omitted C x C tests account for more than half the total tests not carried out.

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 x C and F x D, whereas E x D heterokaryons took about 2 days to initiate growth. B x F positive tests were variable in time of initiation but occurred, on the average, about 5 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 x F combinations, and also D x 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 18-10 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 2 days, whereas 23-56, in tests with 22 B mutants took about 4 days. Both complemented slowly with 60% of the C mutants (an average of 5 days in each case) and would not complement at all with 40% of the C's. To represent these mutants on the complementation map would inevitably require two non-adjacent 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% of the B mutants after an average of 8 days. Although there was insufficient data to characterize completely 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 inog. 89601, have been assayed for the enzymatic ability to convert pyruvic acid to acetoin, by way of acetolactic acid (40). Acetoin was determined by the method of Westerfeld (41). A representative mutant in both the iv-1 and iv-2 groups as well as inog. 89601 displayed substantial activity for these reactions, whereas iv-3 mutants had little if any activity. This evidence indicates that iv-3 mutants are blocked in Step I (Fig. 1.) of the biosynthetic pathway.

An iv mutant, 7110, has been shown by Wilson and Adelberg (42) to accumulate several organic acids, the principal ones being citramalic and \( \gamma, \beta \)-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 Y (37), was crossed to members of each of the five groups and proved to be unlinked or distantly linked to mutants of 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 8.3 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 (10-3), no prototrophs were observed among approximately 2,000 germinated random spores. In this case there was 21% germination. Therefore either represent defects at identical or very closely linked sites. This evidence suggests that the three iv groups represent 3 genetic loci. Two of these (iv-1 and iv-2) are in linkage group Y, about 6.3 map units apart, and both are unlinked to iv-3.
DISCUSSION

General

The most apparent conclusion from the interallelic complementation studies is that all of the iv-2 data and the vast majority of the iv-3 data are compatible with simple linear complementation maps. 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. In addition, the data are in general accord with Woodward's observation (18) that the vigor of heterokaryotic growth between two mutants increases with "distance apart" on the complementation map.

On the basis of these results the hypothesis was 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, in addition, proposed that this pattern also reflects the molecular structure of the enzyme controlled by the locus. The experiments will therefore be discussed with respect to this notion that it is a fundamental property of the gene to specify a particular arrangement or set of subgroups.

Providing 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 \textit{iv-2} mutants, six fell into one subgroup. It is predicted that the number of \textit{iv-2} subgroups will continue to remain small with the accumulation of more \textit{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.

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" (43) and selection (during the experiments by which the mutants were obtained). In phase 94 it has been shown that the spectra of "hot spots" at a genetic locus, induced by unrelated mutagens, show little,
If any, overlap (44). Therefore, the observation that 23 of 27 non-U.V. induced \textit{uv-3} mutants gave the same few complementation patterns observed for U.V. 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.

A few mutants were omitted from the tabulations because their slow growth on minimal medium interfered with the tests necessary for classification. From the tests that could be performed, there was no indication of unusual complementation behavior.

Wagner, Somers and Bergquist (20) 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 side of the complementation map were deemed defective in the reductoisomerase step (Fig. 1.), and those on the other side, 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 4 map units) in the right arm of linkage group V.

On the basis of data presented here a contrasting conclusion has been reached. In this work mutants representing
two completely separate complementation groups (iv-1 and iv-2) appear to be located in linkage group V. The conclusion that the two groups are completely separate is based on the observation that all the 820 tests performed between mutants representing each group were clearly positive within one day, without any indication of ambiguity. One mutant, 13117, used in both studies, was placed on the dehydrase side of their complementation map, but in the present analysis was located in the iv-1 group.

The term "cistron" is often found in the literature associated with reports on complementation phenomena. However, there is some disagreement in its application to different systems. Benzer originally (46) defined the cistron with special reference to the behavior of the r (rapid lysis) mutants in linkage group II of the bacteriophage T4. These mutants each lack the ability to multiply in certain strains of E. coli. Two RII mutants are considered to complement each other if, after simultaneous infection of one of these bacteria with both mutants, progeny bacteriophage are produced. Two mutants are considered non-complementary if, on paired infection, they are defective with respect to this function. Initially Benzer reported that all of these RII mutants belonged to one of two complementation groups. All mutants in one group failed to complement with each other, but each would complement with any mutant of the other group. The mutants in each group proved in recombination tests to represent a continuous sequence of sites in a small segment of the genetic map. Each of the two
segments thus defined was termed a "cistron". Benzer has recently reported (43) weak complementation between mutants that were allocated to the same "cistron". It is not known yet whether a study of this weak complementation will yield a complementation map similar to those obtained in Neurospora. However, it seems clear that it would be most consistent with Benzer's original usage to apply the designation "cistron", in this work, to the complementation group.

Mechanisms

A number of different mechanisms have been proposed to fit the experimental observations associated with interallelic complementation. Before reviewing those involving cytoplasmic interactions it is first necessary to discuss, as a possible explanation, direct somatic recombination between the mutant genomes.

Pateman and Fincham (47) were unable to demonstrate any marker recombination between the vegetative nuclei of heterokaryons. Their evidence could be taken to indicate either the complete absence of genetic interaction among vegetative nuclei or that any such interaction must be far too infrequent to account for the high degree of restoration of enzymatic activity often observed. These findings plus the demonstration of in vitro complementation with mixed crude extracts of mutants (15) argue strongly against somatic recombination as the valid mechanism.
Woodward et al. (13) have speculated that exchange between paired defective RNA templates giving rise to functionally intact templates could account for restoration of enzyme activity. Another very similar theory is that active enzyme protein could be formed from two templates defective at different sites by first "copying" information from one and then from the other. By either of these mechanisms, assuming random exchange or switching, it is predicted that no more than 1/4 of the activity present in wild-type could be restored.

The observation that enzymatic activity achieved in heterokaryons never exceeded this value was thought to lend considerable support. However, in opposition to this type of theory, Woodward (48) has recently shown that mutant proteins, similar in chromatographic properties to the wild-type enzyme, can be used directly to effect in vitro complementation. In addition these theories predict that the restored activity should reside in protein indistinguishable in physical properties from wild-type protein. This is also in direct contradiction to the recent findings of temperature sensitive enzyme in complementing heterokaryons by both Fincham (12) and Case and Giles (17). It is therefore unlikely that a mechanism involving RNA template interaction is involved.

Human hemoglobin molecules are known to be composed of two pairs of polypeptide subunits. The shorthand designation used to describe this arrangement is $\alpha_1 \alpha_2 \beta_1 \beta_2$. $\alpha$ and $\beta$ designate polypeptide chains with different amino acid sequences, whereas $\alpha_1$ and $\alpha_2$ are identical, and $\beta_1$ and $\beta_2$ are identical. At
Another proposed mechanism of complementation is based on the findings of Richards (32). When crystalline bovine pancreatic ribonuclease is treated under specific conditions with the proteolytic enzyme subtilisin, the N-terminal peptide containing 20 residues is split off. This peptide and the residual protein separately have less than 5% of the original activity of the enzyme. When these fragments are brought together under certain conditions, full activity is restored. The active structure is now composed of two peptide fragments held together only by tertiary linkages. To explain complementation on the basis of the above system the presumption is made that complementing mutants must synthesize inactive peptide fragments, which after heterokaryon formation, can join to form active protein. However Yanofsky (23) has reported that complementing mutants as a rule produce protein which cross reacts immunologically with antibodies against wild-type enzyme. Non-complementing mutants do not produce such materials. Since small peptides are not likely to be cross reacting, Yanofsky's data are hard to reconcile with the assumption that the ability to complement depends on fragment formation.

The last of the hypothesis to be discussed is founded on the supposition that enzyme molecules with identical functions tend to be specifically aggregated in vivo. A number of examples of specific protein aggregates have been described. Soyama (53) and Hidea et al. (54) have shown that animal liver glutamic dehydrogenase can be disassociated into at least eight subunits which retain full activity and are therefore probably
identical. Allison (55) has proposed a theory of aggregation to explain the behavior of haptoglobin molecules. Harrison (56) has suggested that ferritin, a protein with a molecular weight of 747,000 should be composed of 24 (or 24n) identical subunits. Studies by Brenner et al. (57) suggest that the tail sheaths of T-even bacteriophages are composed of about 200 repeated protein subunits of approximately 50,000 molecular weight. The head membrane of these bacteriophages is also composed of a large number of repeated subunits with a molecular weight of 80,000. The protein coat of the tobacco mosaic virus has been shown to consist of 2,200 identical subunits of molecular weight 18,000 (58). Present evidence therefore indicates that specific protein aggregation may be a rather prevalent phenomenon. To account for interallelic complementation on this basis it is necessary to assume that in heterokaryons the defective enzyme molecules from the two contributing mutants form a mixed aggregate. In certain cases the two kinds of altered enzyme can interact in the aggregate so as to allow partial amelioration of the defects. One can imagine, as a specific possibility, that close packing of stearically altered proteins forces the molecules into a more nearly normal configuration. In this scheme overlap mutants would be characterized by proteins so stearically altered as to be unable to interact favorably with several different types of defective molecules. A basic difficulty with this type of mechanism is that there is as yet insufficient evidence in support of the assumption that the type of aggregation
described is applicable to soluble enzyme systems. Also bearing in mind the well characterized tertiary structure of myoglobin (59), it is difficult to believe that complementation interactions among molecules of such complexity would be representable by a linear map.

In conclusion, it should be emphasized that all the possible kinds of mechanisms that could be invoked to explain complementation were not exhausted in the above discussion. Only those theories were mentioned which have recently received some attention in the literature. However since one must still consider these to be highly speculative, it is possible that the true mechanism will eventually prove to be basically dissimilar to all of the theories discussed.
BIBLIOGRAPHY


