# STUDIES ON LOCI ASSOCIATED

# WITH CAROTENOGENESIS IN NEUROSPORA

# SOME STUDIES ON LOCI ASSOCIATED WITH CAROTENOGENESIS IN *NEUROSPORA CRASSA*

by

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SCOPE AND CONTENTS:

This thesis proposed to analyse the recombination, complementation and biosynthetic implications of a series of hitherto unstudied carotenoidless mutant strains of *Neurospora crassa* and to confirm the reports of previous authors through analysis of a number of their mutant strains. A new selective technique permitted the fine structure analysis of the locus. Complementation studies with an extensive range of mutants including several recently discovered phenotypes permitted the resolution of new cistronic limits within the locus. A speculative model of the gene products was proposed to embrace the recombination, complementation and biochemical paradigms.

# PREFACE

This thesis describes studies undertaken in the Department of Biology, McMaster University from September 1965 to January 1969. Except where others are specifically mentioned, it consists entirely of my own work. No similar thesis has been submitted at any other university.

My grateful thanks are due to my supervisor Dr. S. F. H. Threlkeld and to Dr. G. J. Sorger and Dr. D. R. McCalla for their advice and encouragement throughout the work. Thanks are also due to the Fungal Genetics Stock Center, Dr. D. D. Perkins, Miss A. L. Shroeder and Dr. A. Radford for the donation of stocks and to the Ontario Department of University Affairs and the National Research Council of Canada for the award of a fellowship and research funds covering the period of the research reported herein. Finally, I wish to thank my typist Miss Mary Marshall for her patience and understanding.

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# INTRODUCTION

## 1. Introductory Remarks

Carotenoids are lipid soluble pigments of unknown function found extensively throughout the plant kingdom. Carotenogenesis or the biosynthetic pathway of carotenoids is only partly understood and very little is known about gene action relating to carotenogenesis.

The common red bread mould "Neurospora" owes its characteristic colour to the carotenoid pigments contained in the vegetative mycelium. The genetics and biochemistry of carotenogenesis may be investigated by studying strains of Neurospora which are mutant in that they are unable to synthesize the full spectrum of carotenoids found in the wild type strain. Carotenoidless mutants occur with a relatively high frequency in Neurospora either spontaneously or after U.V. irradiation. Indeed the first mutant studied in Neurospora was a spontaneous carotenoidless or "albino" (Dodge, 1930a,b) and it was work with this mutant that indicated the potential of the ordered ascus as a source of information on recombination which Neurospora subsequently afforded the science of Genetics.

Carotenoidless mutants in Neurospora possess; growth, germination and fertility characteristics similar to the wild type strains. Two loci associated with carotenogenesis are known in Neurospora, one of which has been divided, though not

unambiguously, into 2 or possibly 3 clustered subunits. The biochemical analysis of these mutants and their related pigments has been attempted several times but a convincing definitive pathway for carotenoid biosynthesis is yet to be resolved.

The succeeding introductory sections survey and comment on the literature related to the genetics and biosynthesis of carotenoids especially that which is relevant to Neurospora carotenogenesis.

## 2. The Genetics of Carotenogenesis in Neurospora

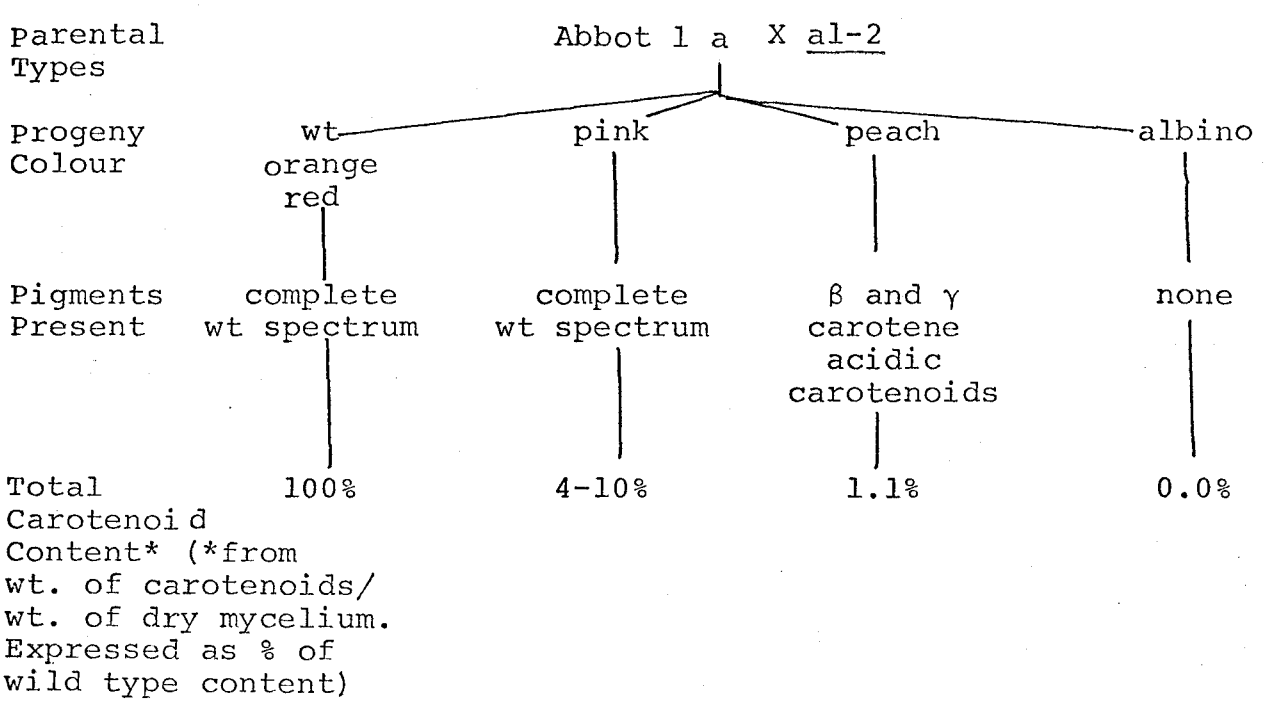
Dodge, working with a mutant for the albino (al) locus (Dodge, 1929; Dodge, 1930a,b) correctly hypothesized that the ascus that shows al segregating at second division represented in fact a crossover that occurred between the centromere which always segregates at first division, and the al locus. This hypothesis is still the fundamental principal for genetic maps from ordered tetrads (Barratt, Newmeyer, Perkins and Garnjobst, 1954).

In a later study (Hungate, 1945) the al locus was divided into two groups on the basis of recombination and complementation data. The first group was typified by al-2 (isolation number 15300) and the second by aurescent (aur isolation number 34508). Heterokaryons formed between mutant strains of different groups complemented to give a wild type (wt) phenotype whereas heterokaryons composed of mutant strains

from the same group had an albino phenotype. She also produced much of the data regarding albino strain germination frequencies, growth rates, fertility and temperature sensitivity. Furthermore, mutants were grown on: extracts from a complementary strain, various vitamin and amino acid media, carotene intermediates, egg yolks, and plant extracts without effect on pigment production. While this study was in progress, one of the albino strains, al-1 (isolation number 4637) was identified as a translocation strain between the right arms of linkage groups I and II (McClintock, 1945, 1955).

A report on a colour intensifier gene that appeared in an Abbot wt cross with al-2 (Sheng and Sheng, 1952) is explained with reference to Figure (i) which described the progeny from such a cross.

Figure (i)



The colours of the four equally occurring progeny types (wt, pink, peach and albino) are seen as genotypes $\underline{c}^+ \underline{i}^+$, $\underline{c}^+ \underline{i}$, $\underline{c}\ \underline{i}^+$ and $\underline{c}\ \underline{i}$ respectively where $\underline{c}$ is the gene related to the ability to synthesize carotenoids and $\underline{i}$, the gene related to the ability to intensify the colouring process. However, none of the foregoing experimental observations has since been confirmed nor does the pattern of inheritance seem to be characteristic of al-2 crosses to Lindegren, St. Lawrence, Oak Ridge or mixed ancestry wt crosses.

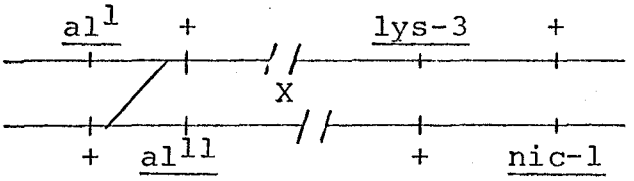
The success of high resolution genetics, where short segments of linkage groups are examined in detail through the study of recombination events between closely linked sites, is predicated upon recognition of the rare recombinant through some selective technique. Such a technique exists for the study of recombination between mutants for biochemical requirements in that half of the recombinants may, unlike the parental types be expected to grow on medium lacking biochemical supplements. The unavailability of such selective techniques for morphological or "visible" mutants has resulted in the avoidance of high resolution studies for these types of mutant strains. Some selective advantage was gained when crosses were made between al strains with adjacent markers for lysine requirement (lys-3), and al strains with an adjacent marker for niacin requirement (nic-1) (Huang, 1964). The lys-3 marker is approximately 16 and the nic-1 marker approximately 15 crossover units (cou) distal to the al region. A comparison

of the frequency of the non-albino recombinants on media supplemented with either lysine or niacin should reflect the relative position or order of the two albino markers, i.e. if the non-albino frequency is highest on the lysine supplemented medium then the al mutant with the lys-3 marker is the proximal al as this configuration requires only one crossover. The relationship between the two possible orders and non-albino frequency is summarized in Figure (ii).

Figure (ii)

ORDER I

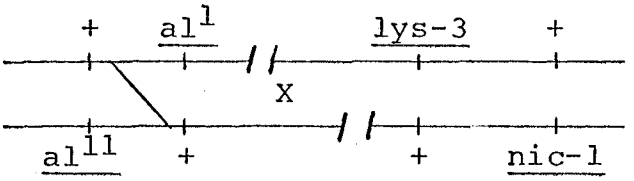
Linkage relationships




Non-albino frequency — higher on lysine supplemented medium

ORDER II

Linkage relationship

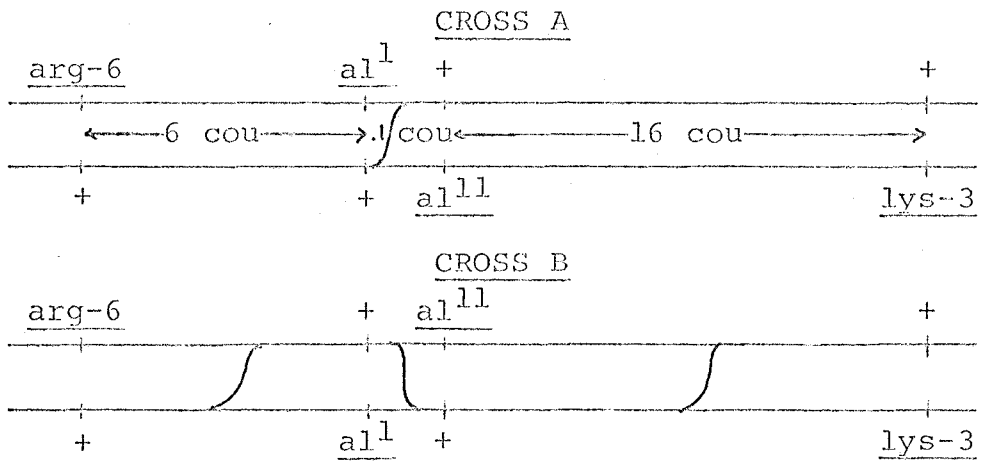



Non-albino frequency — higher on niacin supplemented medium

The al' lys-3 X al" nic-1 type crosses gave a selective advantage of 50% as only one of the parental types was excluded on the selective medium. Huang suggested (1964) that greater selective advantage could be achieved by making the al' X al" cross first with conventional outside markers

(Whitehouse, 1965) then repeating the cross with the outside markers transposed as shown in Figure (iii).

Figure (iii)




If al' was proximal to al" then cross A should produce significantly more (>400X) non-albino recombinants than cross B as the former cross required only one crossover to produce a non-albino versus 3 for cross B. Huang suggested that the selective advantage from such crosses would approach that of nutritional markers although he never tested the hypothesis. Instead he finished his recombination map by determining the recombination frequency through simple al' X al" crosses. He ignored data (Pittenger, 1954) proving the presence of pseudowild types for this region and did not test all his "recombinants" for heterokaryosis. Indeed, heterokaryosis was indicated from his own data directly relating non-albino frequency to plating density i.e. number of spores per plate. Such heterokaryons may be the results of PWT's or hyphal anastomoses of germinating ascospores. In one of the crosses

that he did test, 50% of his non-albino progeny were heterokaryons suggesting that heterokaryosis is a phenomenon to be accounted for and that failure to do so results in an exaggerated distance between the two major complementary groups. In general terms Huang's conclusions (Huang, 1961, 1963, 1964) tended to confirm Hungates evaluation of the dichotomy of the al region.

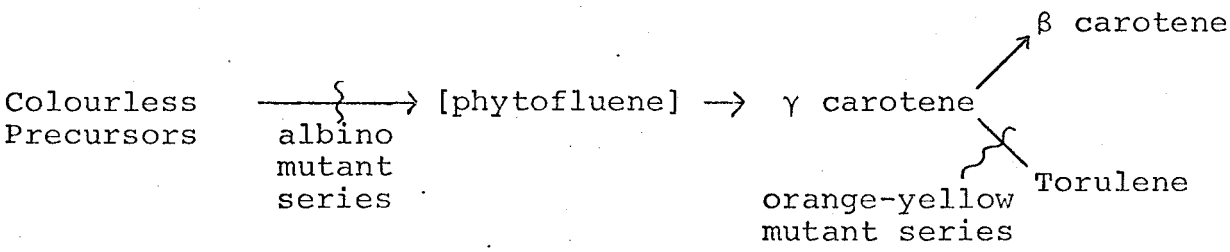
## 3. Genetics of Carotenoid Synthesis in Other Systems

Extensive genetic research has been directed towards developing a tomato with characteristic redness (from lycopene content) and high provitamin A content (β carotene). Research is complicated by at least two carotenogenic systems (Costes, 1965) which are not mutually exclusive. One predominates in the fruit and the other in the leaves. A lesion in one biosynthetic pathway may be masked by the effects of the other pool hence it is the ratio of pigments present in a given tissue that best indicates gene action. A rather complex genetic system has been elucidated by several groups (LeRosen, Went, Zechmeister, 1941; Jenkins and MacKinney, 1955; Tomes, Quackenbush, Nelson and North, 1953; and Tomes, 1967) wherein genes R, I, B and Del control synthesis (in the fruit) of lycopene, prolycopene, β ionone and α ionone respectively.

Microbial carotenoid research is primarily concerned with oxygenated carotenoid derivatives or the photosynthetic implications of carotenoids. One study relevant to this thesis analyzed the pigment mutant series of Rhodotorula rubra

through accumulation studies (Bonner, Sandaval, Tang and Zechmeister, 1946). The mutants were divided into two main series: (1) an albino series which was unable to produce any coloured carotenoids and (2) a yellow series which failed to produce torulene and possessed high levels of γ and β carotene. The mutant series represent blocks or lesions in carotenoid synthesis whose positions are suggested in Figure (iv).

Figure (iv)




(after Bonner et al. 1946 and Simpson et al. 1964)

4. The Carotenoids Present in Neurospora

Neurospora carotenoid identification began with the century (Went, 1901, 1904; van Wisselingh, 1915). Later three Neurospora carotenoids were separated, one of which was tentatively identified as lycopene (van Deventer, 1930).

References to procedures and techniques from various groups (Zechmeister, 1934-1946; and Karrer, 1938-1947) contributed to a monumental work by Haxo (1947-1955) who produced the first comprehensive account of the Neurospora pigments. As part of this report he isolated and characterized a new pigment "neurosporene" which was subsequently found to be a universal

carotenoid. Verified later, (Krzeminski, 1959) the account remained the most authoritative until recent times.

Neurosporoxanthin and spirilloxanthin were shown to be 4-β-apo-carotenoic acid and 3,4 dehydrolycopene respectively (Aasen, 1965) and a new pigment torulene was identified by Jensen (1965). β-Zeacarotene was identified independently by Harding (1968) and Subden (unpublished) completing the list of pigments identified in Neurospora and summarized in Table I.

TABLE I

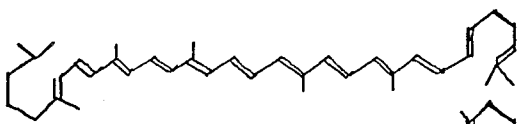
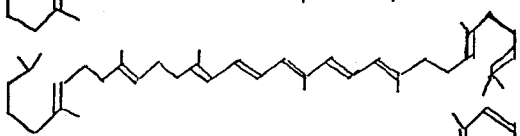
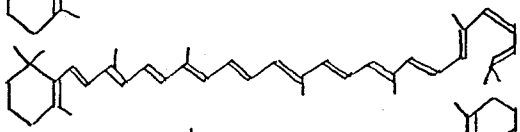
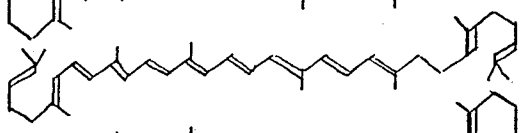
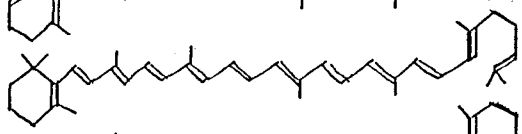
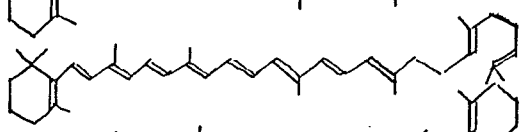
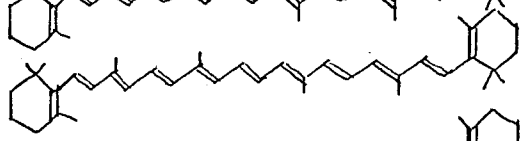
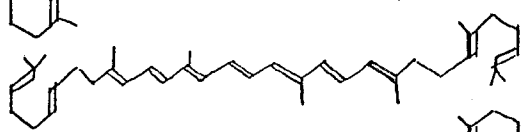
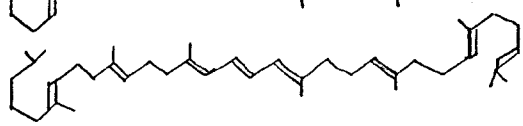
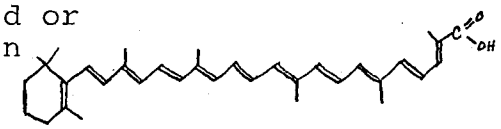
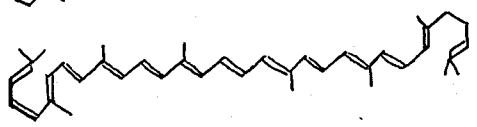
| Pigment | Structure | Reference |
|---|---|---|
| lycopene |  | van Deventer, 1930 |
| phytofluene |  | Zechmeister and Haxo, 1946; Haxo, 1947 |
| torulene |  | Jensen, 1965 |
| neurosporene |  | Haxo, 1947 |
| γ carotene |  | Haxo, 1947 |
| β zeacarotene |  | Haxo, 1947; Subden, 1968; Harding, 1968 |
| β carotene |  | Haxo, 1948 |
| ζ carotene |  | Zalokar, 1954 |
| phytoene |  | Zalokar, 1954 |

TABLE I (continued)

| Pigment | Structure | Reference |
|---|---|---|
| acidic carotenoid or neurosporoxanthin or 4-β-apo-carotenoic acid |  | Haxo, 1947<br>Zalokar, 1957<br>Aasen, 1965 |
| 3,4 dehydrolycopene<br>"spirilloxanthin" |  | Jensen, 1965<br>Haxo, 1947 |

It should be noted that no α-ionone ring structures have been found in Neurospora, e.g. α-zea, δ-, α- or ε-carotenes. Neither have hydroxylated, keto, aldo, oxymethyl, carboxyl or otherwise oxygenated xanthophylls been found save the 35C, neurosporoxanthin. The basic simplicity of the Neurospora pigment range presents an ideal system for carotenogenic analysis.

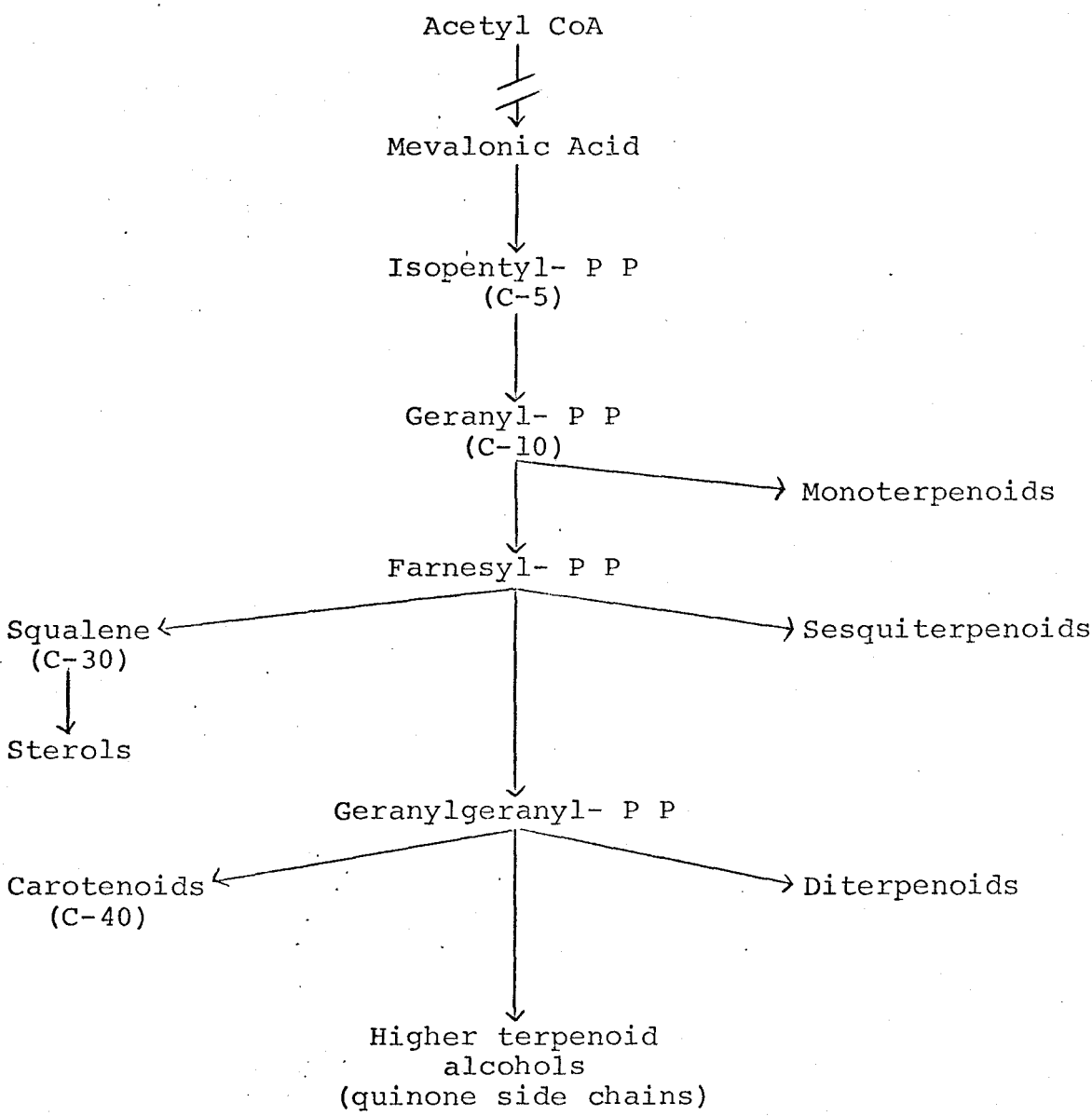
## 5. Biosynthetic Mechanisms

The "Isoprenoid rule" (Ruzicka, 1953, 1959) relates steriod, terpenoid and carotenoid synthesis to the fate of a common 5-carbon unit subsequently identified as isopentyl pyrophosphate. The overall picture for isopenoid metabolism is as seen in Figure (v).

Figure (v)

BIOGENETIC RELATIONSHIP

OF THE ISOPRENOIDS




(after Goodwin, 1965)

It should be noted that the condensation of 2-geranylgeranyl pyrophosphates to yield one phytoene is the first metabolic process specific only to carotenoid biosynthesis, see Figure (v). A more detailed description of isoprenoid metabolism leading to C-40 polyenes or the colourless carotenoids is provided in Figure (vi).

Biosynthetic schemes from phytoene to end products were pre-eminently the concern of the tomato geneticists and biochemists. A stepwise dehydrogenation from colourless saturated precursors to coloured unsaturated end products was suggested (Zechmeister and Sandoval, 1946) and the subsequent discovery of phytofluene and other intermediates led to several elaborations on this theme (Porter and Zscheille, 1946; Porter and Lincoln, 1950; Zechmeister and Koe, 1954; Porter and Anderson, 1962; and Harding, 1968) see Figure (vii)A,D.

Another scheme (Goodwin, 1958; Mackinney, 1952) suggested a parallel synthesis wherein a range of end products would be synthesized directly from a common colourless C-40 precursor, (see Figure (vii)B). Yet another scheme suggested (Bulger, 1966) was that two $C_{20}$ units were partially dehydrogenated then condensed to give a range of end products (see Figure (vii)C).

Figure (vi)

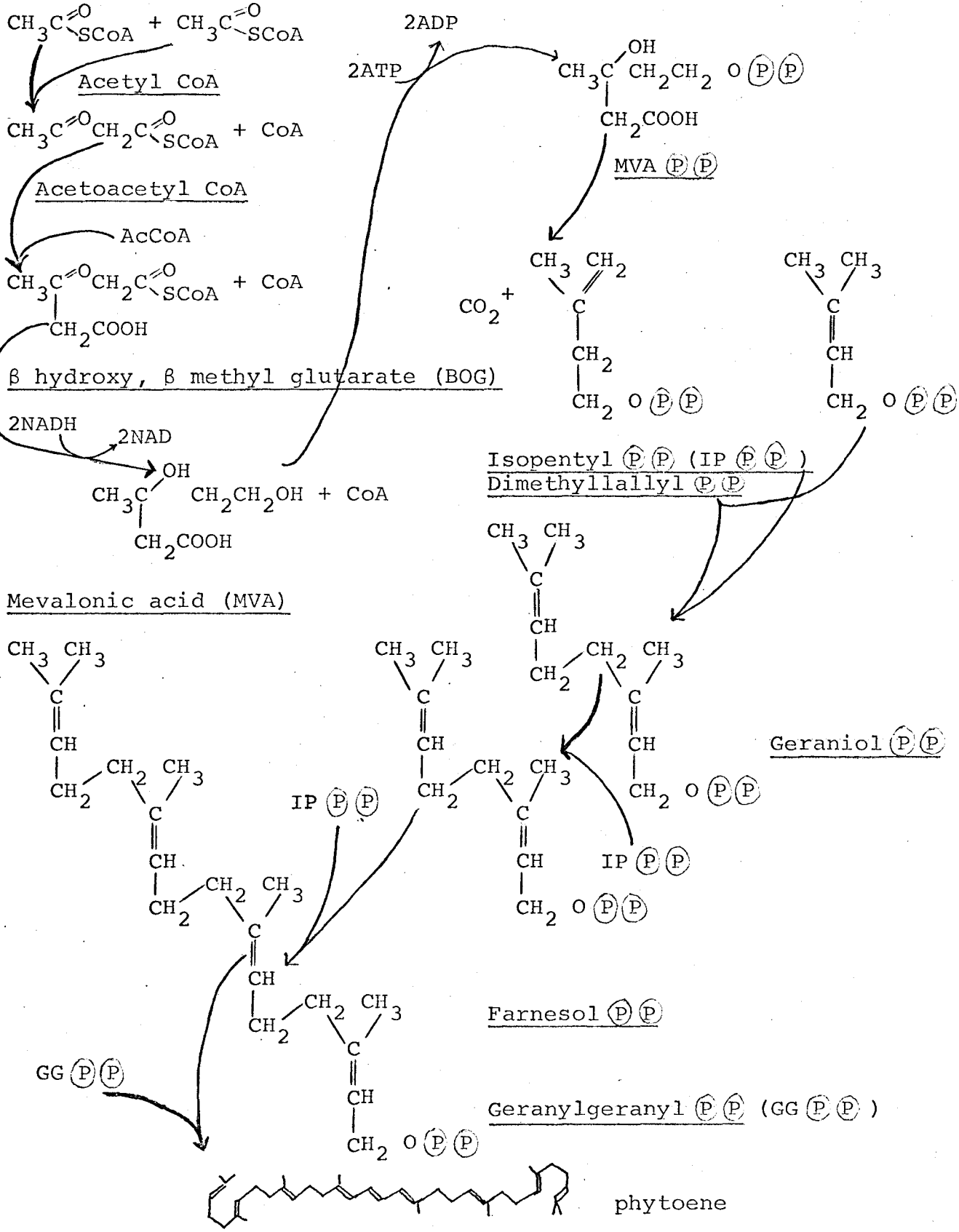
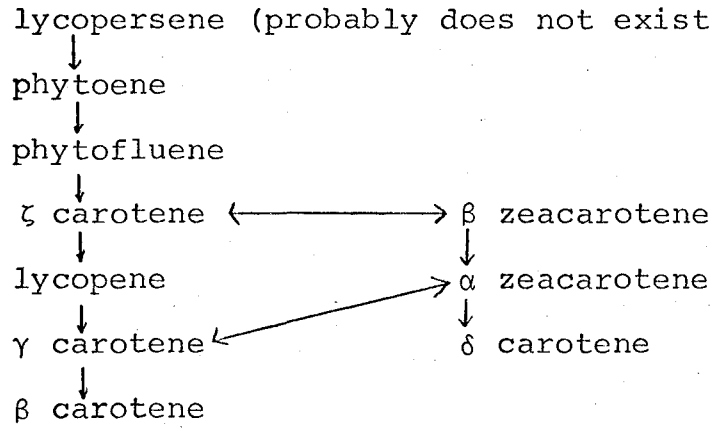
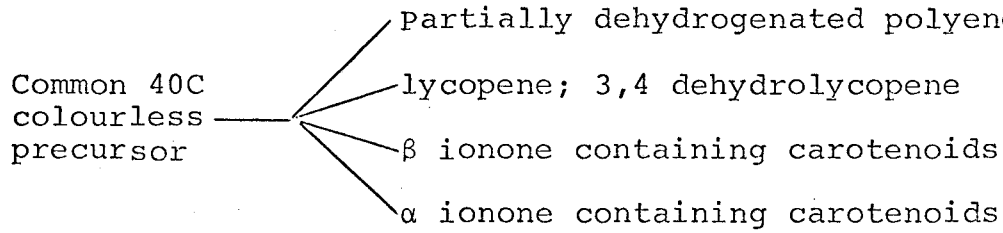

Acetyl CoA
Acetoacetyl CoA
AcCoA
β hydroxy, β methyl glutarate (BOG)
2NADH
2NAD
2ATP
2ADP
MVA (P)(P)
Mevalonic acid (MVA)
Isopentyl (P)(P) (IP (P)(P))
Dimethyllallyl (P)(P)
Geraniol (P)(P)
IP (P)(P)
Farnesol (P)(P)
GG (P)(P)
Geranylgeranyl (P)(P) (GG (P)(P))
phytoene

Figure (vii)

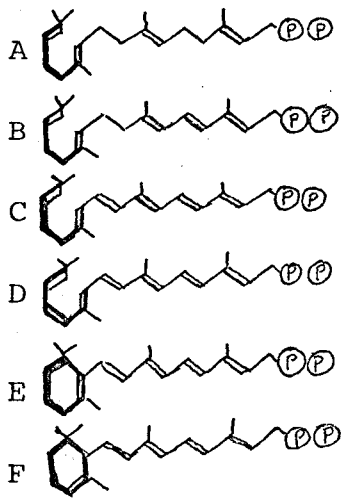
A. Stepwise dehydrogenation (Porter and Lincoln, 1950; Porter and Anderson, 1962).




B. Parallel Synthesis from a 40C Precursor (Goodwin, 1958)



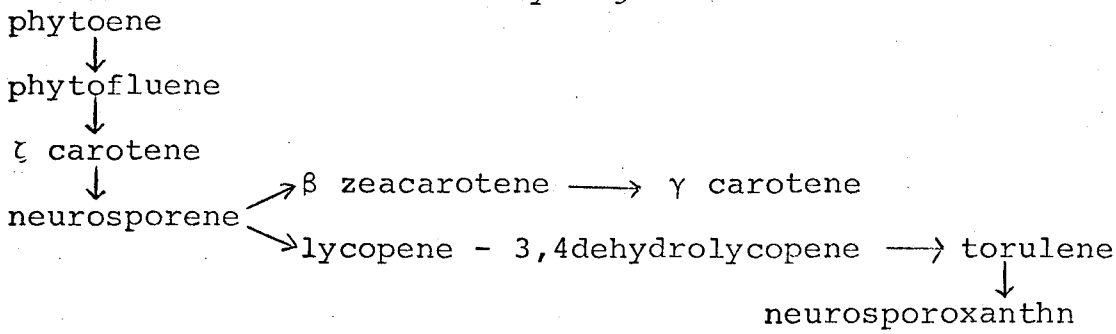

C. Condensation of two 20C units (Bulger, 1966)




| | | |
|---|---|---|
| AA | phytoene | ) |
| AB | phytofluene | ) |
| AB | ζ carotene | ) |
| BC | neurosporene | ) |
| CC | lycopene | ) found in |
| CD | 3,4 dehydrolycopene | ) Neurospora |
| EB | β zeacarotene | ) |
| EC | γ carotene | ) |
| ED | torulene | ) |
| EE | β carotene | ) |
| FB | α zeacarotene | |
| FC | δ carotene | |
| FE | α carotene | |
| FF | ε carotene | |

D. Scheme suggested for Neurospora based on Porter-Anderson (1962).

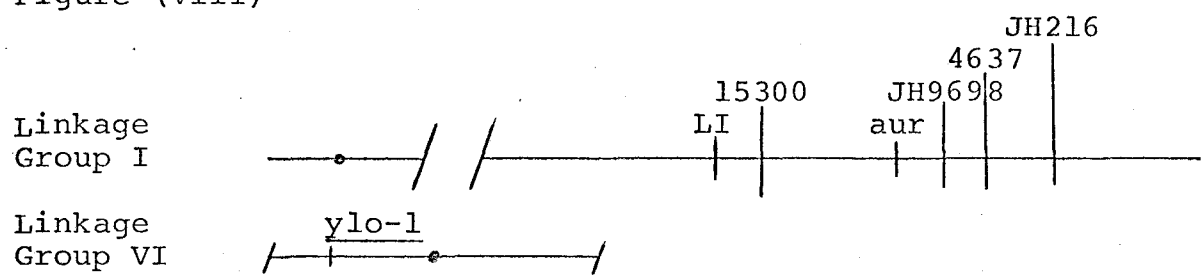
Model of Successive Dehydrogenation




6. Carotenoid Accumulation Studies on Mutant Strains of Neurospora

On the basis of accumulated recombination data (Hungate, 1945; Haxo, 1947; Garnjobst, 1956; Huang, 1964) a linkage relationship may be established as shown in Figure (viii).

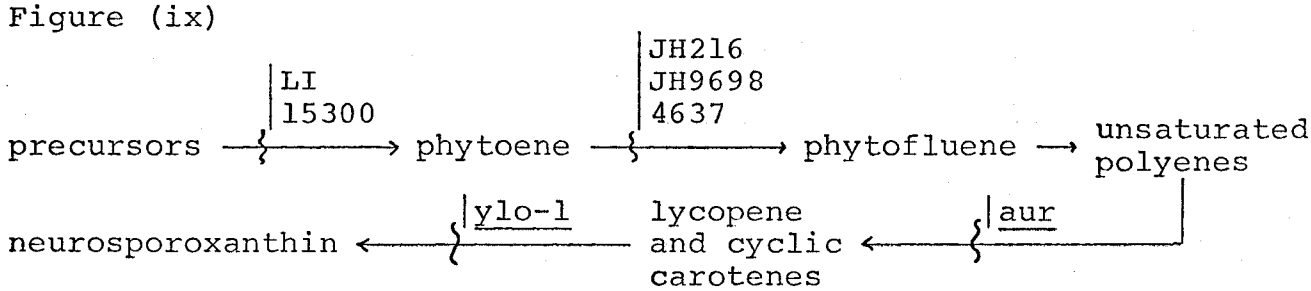
Figure (viii)




The specific blocks or lesions found in the aforementioned mutant strains are positioned in the biosynthetic pathway (Haxo, 1949, 1952) as shown in Figure (ix). This was accomplished by comparing the carotenoids accumulated in the mutant strains to those in the wild type. One assumes that those carotenoids absent in the mutant spectrum are synthesized after the blocks. For many nutritional mutants,

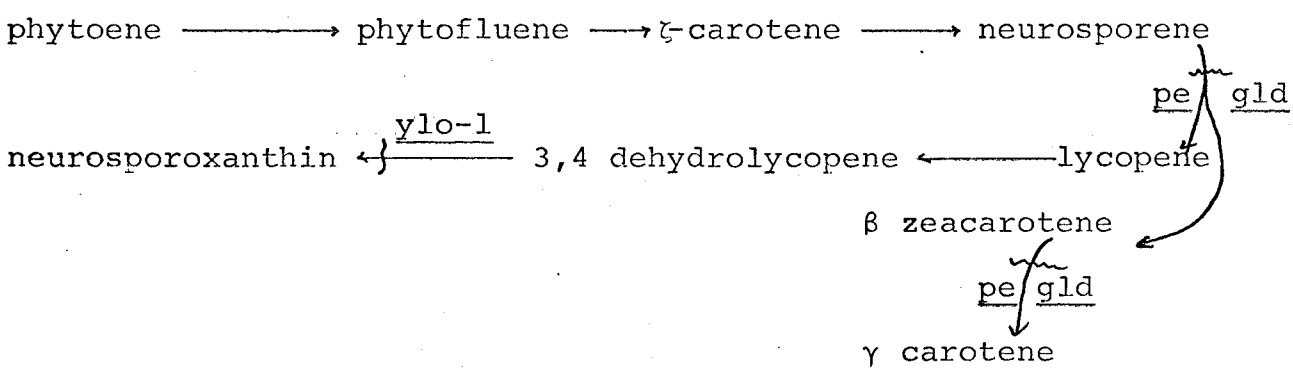
selected intermediates in the biosynthetic pathway are added to the medium which if absorbed, may bypass the effect of the block and restore wt growth. In this manner the activity affected by the mutation can be positioned unambiguously. Unfortunately, though, Neurospora (see Section 2) is not able to take up and incorporate carotenoid intermediates.

Figure (ix)




A recent report (Harding, 1968) with the complete (except β-carotene) Neurospora carotenoid range places the blocks caused by peach (pe), gold (gld), and yellow (ylo-1) as shown in Figure (x).

Figure (x)



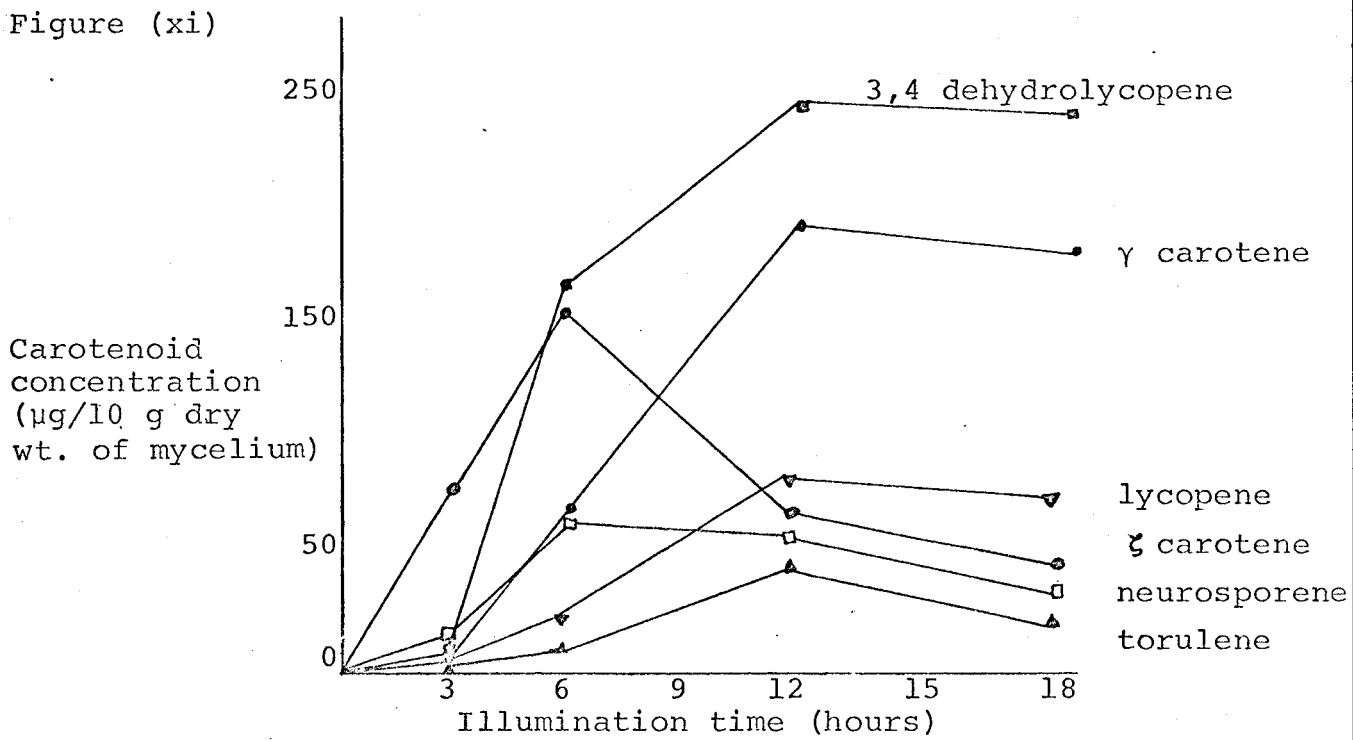

It is unfortunate that the research by the preceding author (Harding, 1968) was on strains that were not truly pigment mutants, i.e. pe is a microconidial strain (Barratt, 1949) and the difference in colour (compared to the wt) is probably

due to the morphological aberrations rather than impaired carotenoid biosynthesis.

7. Time of Carotenoid Synthesis.

The carotenoid concentration at different times after illumination has been used to supplement pathway studies (Harding, 1968; Zalokar, 1954). The rapid decline in the ζ carotene (Figure (xi)) and neurosporene concentrations coincides well with an increase in the neurosporoxanthin, 3,4 dehydrolycopene and γ carotene concentration which is compatible with a possible precursor role for the former pigments. A graph of the changes in carotenoid concentrations is provided for later reference (see Figure (xi)).

Figure (xi)



8. Intent

This investigation was undertaken to resolve the fine structure (high resolution analysis) of the loci associated with carotenoid pigment synthesis. Special concern was to be directed to analysis of carotenoid mutant strains hitherto unstudied, in particular those strains with phenotypes not previously reported. An integral part of the high resolution analysis was the exploration and evaluation of a selective technique for fine structure analysis of morphological or visible mutants strains. This selective technique was described previously in the review of P.C. Huang's recombination data in Section 2.

Complementation experiments were to be performed with all strains to reveal complementation patterns and relate these possibly to gene function.

In addition, studies were initiated to determine just what steps in carotenoid biosynthesis were being affected by the mutations in carotenoidless strains, in particular those strains with previously unidentified phenotypes.

In summary, these studies were begun to analyse the recombination, complementation and biosynthetic implications of a series of newly discovered carotenoidless mutant strains and confirm reports of previous authors working with some previously identified mutant strains.

## 9. Strains Used

The descriptions of the strains used in this study are as follows:

| Marker Designate | Phenotype | Isolation Number | Mating Type | Linkage Group | Ancestry | | Mutagen | Obtained from |
|---|---|---|---|---|---|---|---|---|
| | | | | | Induced In | Backcrossed Into | | |
| wt | wild type | 74A-OR23-1A | A | - | - | - | - | deSerres |
| wt | wild type | 74-OR8-1a | a | - | - | - | - | deSerres |
| al-2 | albino | 15300 | A/a* | I R | L | SL 2 | X | Perkins |
| aur | cream white "aurescent" | 34508 | A/a | I R | L | SL 2 | U.V. | Perkins |
| ylo-1 | orange yellow | Y305304 | A/a | VI L | M | M | M | Perkins |
| ylo-2 | pale yellow | Y256M230 | A/a | - | SL | O | U.V. | Kapuler |
| ylo-3 | orange yellow | Y234M474 | A/a | III R? | SL | SL | U.V. | Kapuler |
| ylo-b | lemon yellow | RES-25-4y | A/a | I R | SL | SL | S | Threlkeld |
| arg-6 | arginineless | 2997 | A/a | L | L | M | U.V. | Perkins |
| arg-5 | arginineless | 27947 | A | II R | L | M | X | Perkins |
| lys-3 | lysineless | 4545 | A/a | I R | L | M | X | Perkins |
| lys-5 | lysineless | DS6-85 | a | VI L | M | M | U.V. | Perkins |
| tryp-1 | tryptophanless | 10575 | A | III R | L | M | X | Perkins |
| tryp-2 | tryptophanless | 75001 | A | VI R | AXL | O | U.V. | Barratt |
| tryp-3 | tryptophanless | C83(td1) | a | II R | E | M | U.V. | Perkins |
| paba | p-aminobenzoic acidless | 1633 | a | V R | L | M | X | Perkins |
| cr | crisp | B123 | A/a | I R | SL | M | U.V. | Maling |
| cys | cysteineless yellow-1 | 84605 Y30539y | A | VI L | L | M | X | Murray |
| ad-1 | adenineless | 3254 | A | VI L | L | M | X | Barratt |
| ad-8 | adenineless | E6 | A | VI L | SL | O | X | Ishikawa |
| al | albino | B102 | a/A | I R | SL | SL | U.V. | Perkins |

| Marker Designate | Phenotype | Isolation Number | Mating Type | Linkage Group | Ancestry | | Mutagen | Obtained from |
|---|---|---|---|---|---|---|---|---|
| | | | | | Induced In | Backcrossed Into | | |
| al | albino | $al^s$ | A | I R | - | M | X | Perkins |
| al | albino | $al^c$ | a/A | I R | M | SL | X | Perkins |
| al | albino | JH216 | a/A | I R | - | - | M | Perkins |
| al | albino | car-1 | A | I R | SL | O | U.V. | Kapuler |
| al | albino | car-7 | A | I R | SL | O | U.V. | Kapuler |
| al | albino | Y256M220 | A/a* | I R | SL | SL | U.V. | Kapuler |
| al | albino | Y256M221 | A | I R | SL | O | U.V. | Kapuler |
| al | albino | Y234M469 | A | I R | SL | O | U.V. | Kapuler |
| al | albino | 80-96 | A | I R | SL | SL | X | Kapuler |
| al | albino | E54R11 | A | I R | SL | O | S | Kapuler |
| al | albino | 7-32 | A | I R | SL | SL | X | Kapuler |
| al | albino | CN 1 | A/a | I R | M | O | S | Robertson |
| al | albino | car-10 | A | I R | SL | O | U.V. | Kapuler |
| al | albino | G2K30 | a/A | I R | - | - | - | Mayo |
| al | albino | Y246M2 | A | I R | SL | O | U.V. | Kapuler |
| al | albino | Y246M3 | A | I R | SL | O | U.V. | Kapuler |
| al | albino | Y254M165 | A | I R | SL | SL | U.V. | Kapuler |
| al | albino | Y256M204 | A | I R | SL | O | U.V. | Kapuler |
| al | albino | Y256M222 | A/a | I R | SL | SL | U.V. | Kapuler |
| al | albino | Y256M231 | A | I R | SL | O | U.V. | Kapuler |
| al | albino | Y256M232 | A | I R | SL | O | U.V. | Kapuler |
| al | albino | Y234M471 | A | I R | SL | O | U.V. | Kapuler |
| ylo-b | lemon yellow | ALS-4-S48-64 | A | I R | SL | SL | U.V. | Schroeder |
| $C^1$ | bright orange | ALS-8-S72 | A | - | SL | O | U.V. | Schroeder |
| al | albino | ALS-14-S73 | A | I R | SL | O | U.V. | Schroeder |
| al | creamy white | ALS-16-S75 | A | I R | SL | O | U.V. | Schroeder |
| al | creamy white | ALS-17-S76 | A | I R | SL | O | U.V. | Schroeder |
| al | albino | ALS-18-S77 | A | I R | SL | O | U.V. | Schroeder |
| ylo-c | pale yellow | ALS-19-S79 | A | I R | SL | O | U.V. | Schroeder |
| al | albino | ALS-20-S80 | A | I R | SL | O | U.V. | Schroeder |

| Marker Designate | Phenotype | Isolation Number | Mating Type | Linkage Group | Ancestry | | Mutagen | Obtained from |
|---|---|---|---|---|---|---|---|---|
| | | | | | Induced In | Backcrossed Into | | |
| al | albino | ALS-21-S81 | A | I R | SL | O | U.V. | Schroeder |
| ylo-c | pale yellow | ALS-22-S82 | A | I R | SL | O | U.V. | Schroeder |
| ylo-b | lemon yellow | ALS-23-S70 | A | I R | SL | O | U.V. | Schroeder |
| al | albino | ALS-24-S71 | A | I R | SL | O | U.V. | Schroeder |
| al | cream white | ALS-25-S72 | A | I R | SL | O | U.V. | Schroeder |
| al | albino | AR-25 | A | I R | SL | O | U.V. | Radford |
| al | albino | AR-8 | a | I R | SL | O | U.V. | Radford |
| ylo-1 | orange yellow | P1193 | A | VI L | SL | O | U.V. | Radford |
| al | albino | 46-11-68 | A | I R | - | - | - | Chalmers |
| pyr-3 pdx-1 | pyrimidineless pyridoxineless | 3730lp H823: 345-5-7 | A | IV R | - | - | - | Threlkeld |

All of the aforementioned strains are available either from the Fungal Genetics Stock Centre in Dartmouth College, New Hampshire or the Perkins collection at Stanford University, Palo Alto, California (except RES-25-47 and 46-11-68).

*A/a means that both mating types have been obtained by backcrosses to Oak Ridge wt's.

In addition, the following Forcing Marker (FM) strains were synthesized by the author. Strains bearing odd FM numbers have adjacent arg-6 nutritional markers while even numbered strains have lys-3 nutritional markers in mixed backgrounds.

| Strain Designate | Pigment Marker Isolation Number | Mating Type | Strain Designate | Pigment Marker Isolation Number | Mating Type |
|---|---|---|---|---|---|
| FM1 | 15300 | A/a | FM21 | AR25 | A |
| FM2 | 15300 | A/a | FM24 | Y256M220 | A/a |
| FM3 | RES-25-4y | A/a | FM28 | G2K30 | A |
| FM8 | RES-25-4y | A/a | FM30 | Y246M2 | A |
| FM9 | 34508 | A/a | FM31 | ALS-19-S79 | A/a |
| FM10 | 34508 | A/a | FM32 | ALS-19-S79 | a |
| FM11 | JH216 | a | FM33 | ALS-4-S48-64 | A |
| FM12 | JH216 | a | FM35 | ALS-23-S70 | A |
| FM13 | $al^c$ | A/a | FM36 | ALS-23-S70 | a |
| FM14 | $al^c$ | a | FM39 | 80-96 | a |
| FM15 | car-1 | a | FM41 | ALS-22-S82 | A/a |
| FM16 | car-1 | a | FM43 | Y256M232 | A |
| FM17 | Y254M165 | a | FM45 | 46-11-68 | A/a |
| FM18 | Y254M165 | A/a | FM48 | ALS25 | A |
| FM19 | Y234M471 | A/a | | | |
| FM20 | Y234M471 | A/a | | | |

Symbols Used

SL - St. Lawrence
O - original
L - Lindegren
M - mixed
E - Emerson

U.V. - ultraviolet irradiation
X - X-ray irradiation
S - spontaneous
M - mustard
A - Abbott

10. Media

The compositions of the trace element solution (TE), inorganic salt solution (4X), various media and supplement concentrations are as follows:

Trace Element Solution (TE)

| | |
|---|---|
| $Na_2B_4O_7.10H_2O$ | 0.01 g |
| $CuSO_4.5H_2O$ | 0.1 g |
| $FePO_4.2H_2O$ | 0.2 g |
| $MnSO_4.4H_2O$ | 0.02 g |
| $ZnSO_4.7H_2O$ | 2.0 g |
| $NaMoO_4.2H_2O$ | 0.02 g |
| Distilled $H_2O$ to | 250 ml |

(Westergaard and Mitchell, 1947)

Inorganic Salt Soltuion (4X)

| | |
|---|---|
| $KNO_3$ | 4.0 g |
| $KH_2PO_4$ | 4.0 g |
| $MgSO_4.7H_2O$ | 2.0 g |
| *$CaCl_2$ | 0.2 g |
| NaCl | 0.4 g |
| Biotin | 16.0 µg |
| TE | 1.0 ml |
| Distilled $H_2O$ to | 1000 ml |

(Westergaard and Mitchell, 1947)

*A substitution of 0.2 gm of $CaCl_2$ for 0.4 gm $CaCl_2.6H_2O$ as stated in the original formula.

Media

| Constituent | Liquid Medium | Crossing Medium | Vegetative Growth Medium | Test for auxotrophy | Assay for prototroph frequency |
|---|---|---|---|---|---|
| carbon source | 2.0% glucose | 2.0% sucrose | 2.0% glucose | 0.4%sorbose 0.2%sucrose | 2.0%sorbose 0.5%fructose 0.5% glucose |
| agar | NIL | 1.5% | 1.5% | 2.0% | 2.0% |
| 4X | 250ml/l | 250ml/l | 250ml/l | 250ml/l | 250ml/l |
| $H_2O$ | to 1000 ml | to 1000 ml | to 1000 ml | to 1000 ml | to 1000 ml |

Nutritional Supplements

| Supplement | Concentration |
|---|---|
| L arginine.HCl | 300 mg/l |
| L lysine.HCl | 200 mg/l |
| L tryptophane | 80 mg/l |
| L-cysteine | 80 mg/l |
| L adenine | 80 mg/l |
| p-aminobenzoic acid | 80 mg/l |
| pantothenate.Ca | 80 mg/l |

There was frequently a problem of an unknown nature wherein random preparations of lysine supplemented media would not gel.

11. Solvents and Chemicals

All chemicals and solvents used were of analytical reagent grade except the solvents for the final neutral pigment preparation (see flow diagram), and the developing of the chromatographic column in which case "spectroanalyzed" hexane (Fisher Scientific Company) was used. Special care was taken (redistillation) to ensure the quality of the solvents as some impurities tended to absorb in the 300-400 mμ range seriously hampering spectrophotometric analysis. Impurities also distorted the chromatograms. Light petroleum ether, b.p. 60-71°C, predominantly hexane, was found to be the most suitable solvent for all the preliminary extractions.

12. Chromatography and Spectrophotometric Apparatus

The chromatographic tubes were pyrex, ID 10 mm with a length of 300 mm and a coarse porosity fritted disc sealed into the inner member of a $ joint which fits the outer member of the packing column. This was removed for extrusion of the column.

The actual column was a mixture of acid-washed celite or diatomaceous earth and Fisher 80 to 200 mesh absorption alumina in a w/w ratio of 1:1. It was packed dry under a vacuum and great care was taken to ensure a constant density. On top of the column approximately 3 cm of $Na_2SO_4$ was placed to: a) remove all particulate impurities, b) to remove all polar inclusions, c) reduce the surface area of the test material, and d) to take up the disruptive influence caused by an uneven application of the test material allowing for more even handing.

A Beckman model DB spectrophotometer was used for the spectrophotometric analysis of the pigment fractions. A quartz cuvet was used for all determinations.

13. Abbrevations and Conventions Used

PWT - pseudowild type, a strain disomic for a given linkage group

TT - tetra type tetrad

NPD - non parental ditype tetrad

PD - parental ditype tetrad

wt. - weight

wt - a wild type Neurospora strain

wt p - a wild type prototroph. The term is used specifically to distinguish between an albino prototroph and one with the full carotenoid spectrum.

FM - Forcing Marker. This refers to a carotenoidless strain that also bears a linked marker for arginine or lysine auxotrophy.

al' or al" - any two heteroallelic albino strains

rec gene - a gene controlling the recombination frequency. $rec^+$ has low and $rec^-$ has high recombination values

cou - cross over units. A measure of recombination map distance

MT - mating type

MeOH - methanol

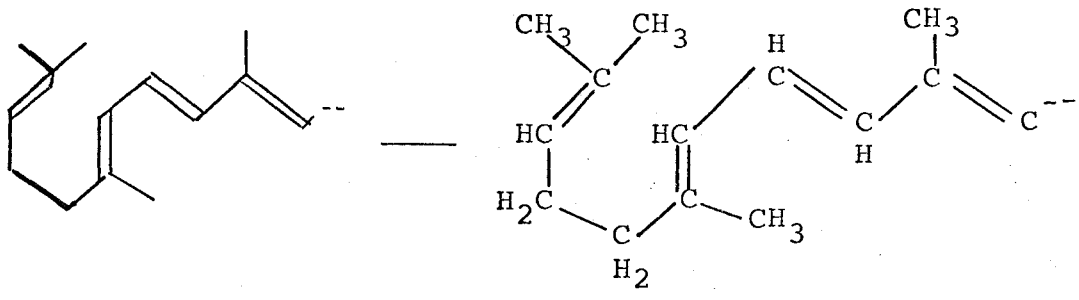
w/v - weight by volume

OR (ORA OR wt ) - an Oak Ridge strain

TE - trace element solution

4X solution - a solution of inorganic salts used in Neurospora media by Westergaard and Mitchell (1947)

GGpp - geranylgeranyl pyrophosphate



14. Crosses

Sexual reproduction in Neurospora is initiated when a certain ratio of nitrogen to carbon is attained in the medium (Hawker, 1957). Supplements like those used in this study (arginine and lysine the diamino-amino acids) tend to increase the nitrogen concentration of the medium and extend the time required for the optimal N/C ratio to be attained. Accordingly, in this study, the age of the maternal or protoperithecial parent was increased from the recommended 4-6 days (Catcheside, 1951) to 10-14 days before inoculation by the male or conidial parent.

Arginine in the medium prevents lysine uptake by Neurospora even though a given strain may require lysine and not arginine for growth (Doermann, 1947). To avoid this inhibitory effect the protoperithecial parent was always the lys FM strain and the conidial parent the arg FM strain.

The conidial suspension prepared from the arg FM strain contained, 4X salts to prevent osmotic shock to the conidia and tween 80 (0.01%) to make the conidia more wettable (Griffiths, personal communication).

Strains producing 30% or more hyaline spores (e.g. ylo-2 - Y256M230), were disregarded as this is generally

accepted as an indication of chromosomal aberrations which tend to reduce the reliability of the recombination data.

15. Ascospore Isolates

Ascospores were scraped from the test tube wall of a 4 week old cross with a sterile wet steel loop and spread across the surface of a block of 4% agar. Individual ascospores were removed on a sterile tungsten needle and transferred to a small tube (10 X 75 mm) containing 1 ml of supplemented glucose medium. Isolates were heat shocked at 60°C for 45 minutes then incubated for one week then the pigmentation was scored.

16. Forcing Marker (FM) Strain Selection

From + al X arg-6 + and al + X + lys-3 crosses ascospore isolates exhibiting albino phenotypes were tested on sorbose minimal (see Section 10) and complete media. Growth was scored after 24, 48 and 72 hour periods. Strains that gave evidence of impaired growth ("leaky mutants") were rejected, as complete auxotrophy was required for the selection of recombinants in the fine structure analysis.

The doubly marked FM strains (al lys-3 or al arg-6) selected, were then retested on sorbose minimal media and in addition tested for mating type.

17. Wild Type and Prototroph Determinations

Ascospores removed from the FM X FM crosses were transferred to 50 ml conical flasks containing 20 mls of a 0.1%

agar solution. The agar increased the viscosity preventing spore precipitation which facilitated spore concentration estimations. From 3000 to 4000 spores from each cross was considered optimal (see Discussion). The spores were heat shocked in the conical flasks, transferred to 500 ml of minimal sorbose medium at 45°C, stirred, then poured into plates. The plates were incubated under intense fluorescent lights (G.E. F96-T12 C.W. 97 at 35 cms) for 14 days at 25°C then scored visually as either wild type or albino prototrophs.

In order to distinguish between phenotypically wt heterokaryons (either from hyphal anastomoses of ascospore germlings or from pseudowild types) and genotypically wt recombinants, a conidial analysis was performed. To preclude data from simple contamination from other colonies on the original plate, the initial colony was subcultured in a small tube from which a conidial suspension was taken for plating on sorbose complete medium (arginine plus lysine). In many cases a further conidial suspension was taken from the preceding plates (using 36hr old isolates) and again plated on sorbose complete medium.

## 18. Low Resolution Studies

RES-25-4y (ylo-b) arose spontaneously as a single spore isolate from a cross H823:345-5-7 X 74-OR23-1A (Threlkeld, unpublished). The pattern of inheritance through three consecutive backcrosses to wt established ylo-b as a single

allele. Ylo-b appears lemon yellow until quite mature at which time reddish pigments are detected in the terminal conidia.

Tetrad analysis was performed on crosses to ylo-1, ylo-2 and ylo-3 to determine possible linkage relationships. Alcoy mapping (Perkins, 1966) was attempted but three colour markers with three instances of hypostasis made results unacceptable. Tester strains with markers representing five of the eleven known linkage group arms were used in the first half of the screening experiment to determine linkage relationships. Indicator markers on the tester strains were approximately half the distance from the centromere to the most distal marker for maximal linkage detection.

## 19. High Resolution Studies

In order to establish confidence in the ability of the selective technique as suggested by Huang (see Section 2) to yield reliable recombination data a series of test crosses were made in which three pigment mutants of independent origin and varying phenotype (al-2, aur, ylo-b) were mated in all possible combinations (Phase I) and the observed results compared with those predicted.from the low resolution results.

As further evidence of the selection technique validity, FM strains using al markers whose linkage relationships had been previously ascribed (Hungate, 1945; Huang, 1964) were crossed (Phase II) and the results compared to the published linkage data.

Succeeding the previously described testing programme all of the unmapped al strains with both FM derivatives e.g. al' arg-6 and al' lys-3 were crossed in all possible combinations (Phase III). Phase IV consisted of crossing singly marked al strains i.e. either al arg-6 or al lys-3 to all of the strains mentioned in Phases I, II and III and it incorporated the sum total of this recombination data into a single composite recombination map.

Finally additional crosses were made to establish the linkage relationships of P1193 (a ylo-1 allele). Selection in these crosses was restricted to locating the wt's amongst prototrophic recombinants between the distal markers ad-8, lys-5 or cys-1 and proximal marker tryp-2, all on linkage group VI.

It should be noted that the primary intent was to determine the linkage relationship with respect to order and that map distances would be used to place mutant sites only when sufficient information was not forthcoming from the ordering technique. Interallelic recombination map distances are not consistently additive (Case and Giles, 1958, 1964).

## 20. Complementation Tests

As an extension of Phase II it was necessary to confirm the published complementation data (Hungate, 1945; Huang, 1964). To do this a variety of techniques were employed: 1) incorporating forcing markers (Catcheside, 1958),

2) nuclear (conidial) input ratio gradients (Huang, 1961), and 3) backcrosses to common wt's to exclude heterokaryon incompatibility gene effects (Garnjobst, 1953, 1955; Garnjobst and Wilson, 1956).

The majority of complementation tests (with Phase I, III, IV and unmarked strains) consisted of mixing one drop of conidial suspension from each "parental" strain on glucose minimal medium in a small tube. The tubes were then maintained under intense fluorescent illumination for 15 days before scoring.

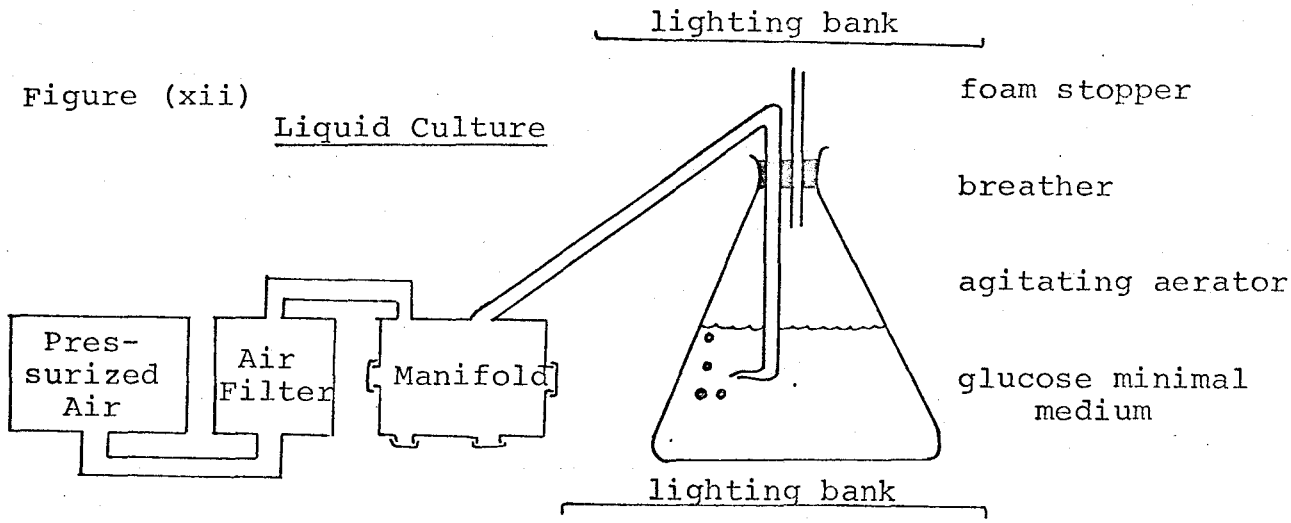
Additional complementation data came from analysis of phenotypically wt colonies from the recombination studies, which upon further analysis proved to be complementing heterokaryons. Pseudowild types were included in this class.

Visual identification of positive complementation was difficult only with ylo-b type + ylo-b type complementation. Conidial suspensions of suspected wt heterokaryons of this sort were plated out on complete medium making colour discrimination in most cases reliable. Inconsistent or unsatisfactory results were obtained by analysis of absorption spectra of heterokaryon extracts to determine positive complementation. This was due probably to nuclear ratio variations.

21. Liquid Cultures

Three ml of conidial suspension was added to 3 l of glucose medium in 4 l erlenmeyer flasks or 1 litre of medium in 2 l low form flasks. Both types of flasks were aerated with filtered air and illuminated as shown in Figure (xii).

Figure (xii) Liquid Culture




The apparatus described in Figure (xii) was autoclaved *in toto*. A drop of tween 80 was added to the medium to keep the mycelium wet, decreasing conidiation and the problems of airborne conidia.

The aeration rate was critical and was adjusted daily. Too little agitation increased colony size which reduced the effective surface area exposed to the light.

Cultures were illuminated by two lighting banks each of which contained 16 fluorescent lamps estimated to have 150 to 200 foot candle power each. The lights produced little heat so a constant temperature of 25°C was maintained with no difficulty. The fluorescent lights were found adequate to initiate the photoactivation process for carotenoid synthesis which is reported to be in the region of 450-488 mμ (Zalokar, 1955).

## 22. Extraction and Purification of Carotenoids

The general procedure for extraction follows that developed by Haxo (Haxo, 1949) and elaborated by subsequent

workers (Zalokar, 1954, 1955, 1957; Krzeminski, 1959; Aasen, 1965; Jensen, 1965; and Harding, 1968).

To prepare the mycelial mass for extraction the living liquid culture was strained and wrung out in cheesecloth until fairly dry. It was then covered in acetone and fragmented in a Waring blendor for 10 minutes producing a thin slurry. The slurry extraction was accomplished through 5 successive washings with a 1:9 (v/v) mixture of methanol (MeOH) and acetone or until no colour remained in the slurry. The slurry and MeOH acetone extract was separated each time in a 3 l separatory funnel. The extract was then filtered through a fine porosity fritted disc in a glass funnel.

Separation of the acidic and neutral pigments was accomplished by a subsequent series of three extractions with analytical grade hexane. These extractions were continued if the third hexane extract showed colour.

To eliminate the fatty acids which tended to plug up the chromatographic columns, the epiphasic layer was reduced in a volume in a rotary evaporator then saponified with an alkaline solution (KOH:MeOH:$H_2O$, 20:90:10, w/v/v). Some of the epiphasic carotenoids that contaminated the hypophasic layer were removed by a subsequent extraction with hexane.

To obtain the acidic pigment in solution the hypophasic fraction was reacidified with 1/5 of an equal volume of 8N acetic acid in hexane. On the fifth day of reacidification, the upper or pigmented layer was decanted with as little agitation

as possible. The acidic pigment solution was then reduced to near dryness and redissolved in 10 ml of petroleum ether or hexane. Hypophasic fractions left longer than 5 days exposed to light turned jet black. The nature of this reaction is completely unknown.

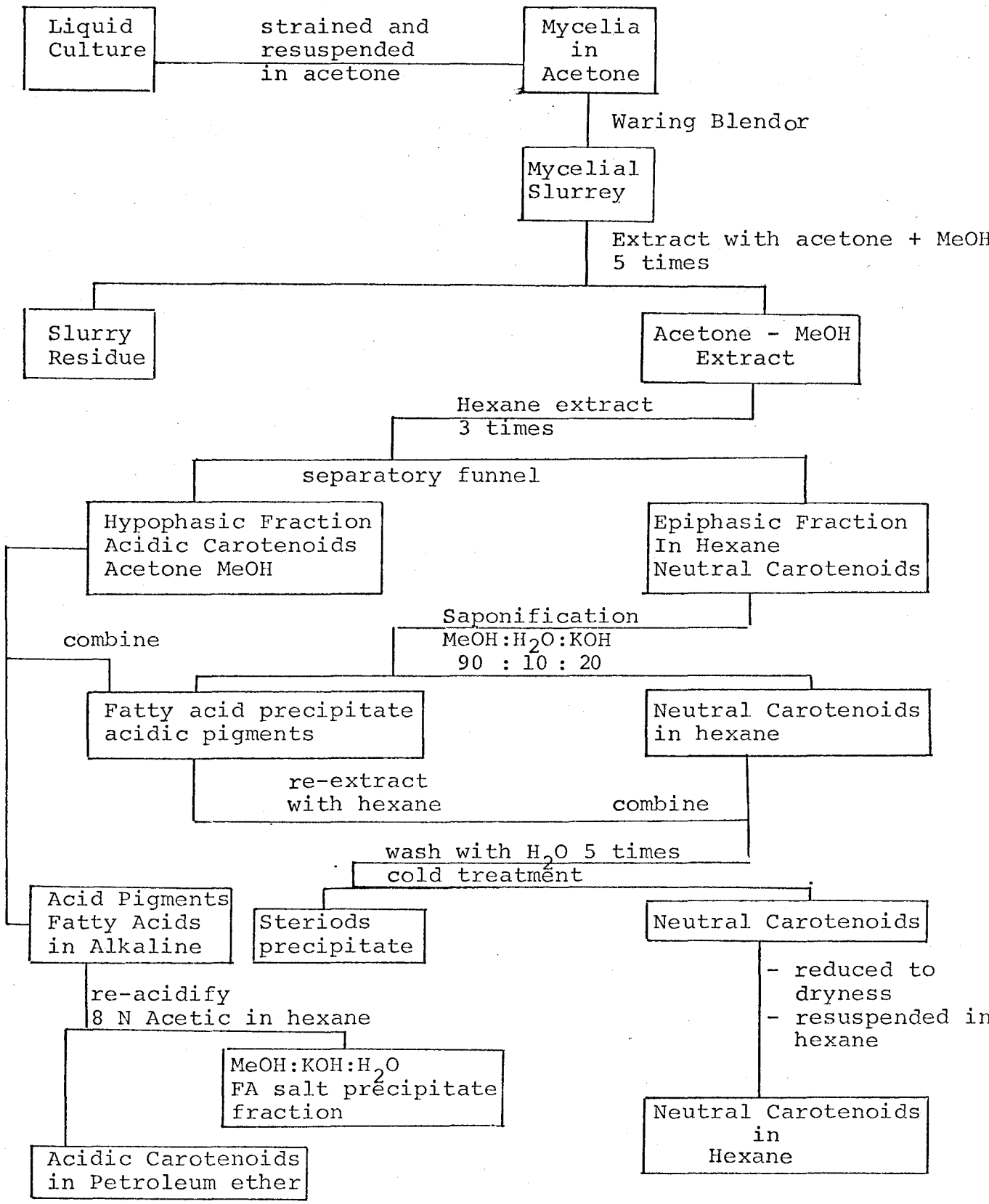
The epiphasic fraction was washed vigorously with distilled water 5 times. This rigorous treatment was necessary to remove all traces of acetone. Clean classware was used for each washing. Steroids were removed simply by chilling the epiphasic fraction overnight at -17°C and percolating the solution in the morning through a 3 cm alumina column to remove the precipitate. The resultant preparation was then reduced to dryness and resuspended in spectroanalyzed grade hexane (Fisher Scientific Co.) and was ready for chromatography. The foregoing procedures are summarized in the Flow Diagram for Extraction and Purification, Figure (xiii).

## 23. Chromatography and Spectral Analysis Procedures

Approximately 3 ml of the pigment extract solution was applied to the column (as described in Section 12) slowly through a drawn Pasteur pipette. When the level of the sample had dropped to the bottom of the $Na_2SO_4$, 1 ml of hexane was added. In turn, when the hexane was adsorbed onto the celite alumina the $Na_2SO_4$ was colourless and the column was completely filled with hexane and maintained replete for the entire experiment.

Figure (xiii)

Flow Diagram for Extraction and Purification of Neutral and Acid Carotenoid Pigments



The development of the column was observed by following the descent of a striking U.V. fluorescent band identified later as phytofluene. Upon closer scrutiny a very faintly fluorescent band is detectable immediately preceding phytofluene. These two polyene bands were collected manually in the dark with U.V. lighting. The fast running carotenoids, β-carotene, β-zeacarotene and ζ-carotene bands were followed visually, collected manually and immediately rechromatographed on a 15 cm column to increase resolution of the band limits. The slow moving bands γ-carotene; neurosporene lycopene - torulene and 3,4 dehydrolycopene were cut out of the extruded column and resuspended in hexane-acetone-MeOH (90:5:5, v/v/v). The slow band solutions were then centrifuged to remove the column material, reduced in volume in a rotary evaporator and resuspended in spectroanalyzed hexane.

All bands collected were kept in sealed tubes at -17°C until the absorption spectra were determined. Best results came if the analysis of absorption spectra immediately succeeded chromatography. Care was taken to minimize the extrusion time as some of the carotenoids are unstable in air (see Table II).

Difficulties were encountered chromatographing the hypophasic fraction so the unchromatographed material was spectroanalysed and found moderately successful.

Pigment concentrations in cuvets from carotenoids displaying dilute bands on the column were adjusted until

the major absorption maximum approached an absorbance of .85. Spectra were then determined and compared to literature values (see Introduction, Section 4). No attempt was made to compute the absolute quantities present as it was primarily qualitative data that was required for the identification of the range of pigments produced by mutant strains. The concentrations of individual carotenoids from mutant strains was determined as a fraction of that found in the wt strain as it was the relative production that was significant. The concentrations in Figure (xxvii) were calculated according to the expression $A = \varepsilon C$ where A is the absorbance in an optical cell of path length 1 cm, $\varepsilon$ the extinction coefficient (from Jensen, 1965) and C the concentration in moles per litre.

Table II

Relative Stability of Pigments*

| Pigment | Relative Stability |
|---|---|
| phytoene | moderate |
| phytofluene | moderate |
| ζ carotene | unstable |
| neurosporene | unstable |
| lycopene | unstable |
| 3,4 dehydrolycopene | no information |
| β zeacarotene | no information |
| γ carotene | moderate |
| β carotene | stable |
| torulene | no information |
| 3,4 dehydrolycopene | no information |

*For carotenoids in petroleum ether, diffuse light at 25°C.

Legend

unstable - 10-25% isomerization in 24 hours

moderate - 4-10% isomerization in 24 hours

stable - <4% isomerization in 24 hours

(from Weedon, 1965)

## RESULTS

### 24. Low Resolution Analysis of RES-25-4y (ylo-b)

The mutant ylo-b was identified as a discrete allele as a result of three backcrosses to wt 74A-OR23-1A, each generation giving a 1:1 ratio for ylo-b and its wt allele. Ylo-b was unchanged phenotypically as a result of these crosses.

The next step was to test for possible allelism to ylo-1 (Y30539y). Accordingly tetrads were taken from a ylo-b X ylo-1 cross, the results of which are shown in Table III.

TABLE III

Tetrad Analysis From ylo-b X ylo-1 Cross

| Phenotype | Postulated Genotype | No. in Class | Tetrad Type* |
|---|---|---|---|
| ylo-b<br>ylo-b<br>ylo-1<br>ylo-1 | ylo-b +<br>ylo-b +<br>+ ylo-1<br>+ ylo-1 | 4 | Parental Ditype (PD) |
| ylo-b<br>ylo-b<br>+<br>+ | ylo-b ylo-1<br>ylo-b ylo-1<br>+ +<br>+ + | 3 | Non Parental Ditype (NPD) |
| ylo-b<br>ylo-1<br>ylo-b<br>+ | ylo-b ylo-1<br>+ ylo-1<br>ylo-b +<br>+ + | 9 | Tetratype (TT) |

| Phenotype | Postulated Genotype | No. in Class | Tetrad Type* |
|---|---|---|---|
| ylo-b / ylo-l / + / ylo-b | ylo-b ylo-l / + + / ylo-b + | 6 | Tetratype (TT) |

*Nomenclature according to Pasher 1918.

Three primary conclusions were drawn from the data of Table III namely: 1) ylo-b and ylo-l are on separate linkage groups or possibly very far apart (48cou) on the same linkage group, based on calculations from the relationship:

$$\text{map or recombination frequency} = \frac{1/2\ TT + NPD}{\text{Total number of Tetrads}} \times 100\%$$

$$= \frac{1/2\ (15) + 3}{22} \times 100$$

$$= 48\% \qquad \text{(Srb, 1952)}$$

2) ylo-b unlike ylo-l is not adjacent to the centromere (ylo-l-centromere distance = 6 cou, Baratt, Newmeyer, Perkins and Garnjobst, 1954). The number of asci segregating at second division for ylo-b was 15 and using the relationship:

$$\text{Centromere Distance} = \frac{\text{No. of 2nd division asci}}{\text{Total no. of asci}} \times \frac{1}{2} \times 100$$

$$= \frac{15}{22} \times \frac{1}{2} \times 100$$

$$= 34 \text{ cou} \qquad \text{(Srb, 1952)}$$

A centromere distance of 34 cou can be obtained for ylo-b.

3) Backcross to ORA results indicate that a ylo-b ylo-l strain exhibits a phenotype characteristic of ylo-b strains, the

ylo-b strain was regarded as epistatic to ylo-1.

Presuming that the ylo-b marker was unlinked to the ylo-1 marker a procedure to screen for linkage was conceived such that certain test marker strains were selected with markers equidistant from the centromere to a distal marker on the same linkage group not more than 50 cou away. A ylo-b strain was crossed to each test marker strain and linkage to a test marker detected after a statistical analysis of the recombinant class frequencies. Five of the eleven arms of the seven linkage groups (see Figure (xiv)) were screened in the first half of the experiment and the results tabulated in Table IV. The low probability (p) for independent assortment for ylo-b and cr or A/a indicate that ylo-b is associated with linkage group I. From the tetrad analysis of the preceding paragraph it was known that ylo-b was approximately 34 cou from the centromere so three further crosses were made with test strain markers 30-40 cou from the centromere namely:

1. cr al-2 + X + + ylo-b
2. arg-6 al-2 + X + + ylo-b
3. + aur + X cr + ylo-b.

Applying the relationship:

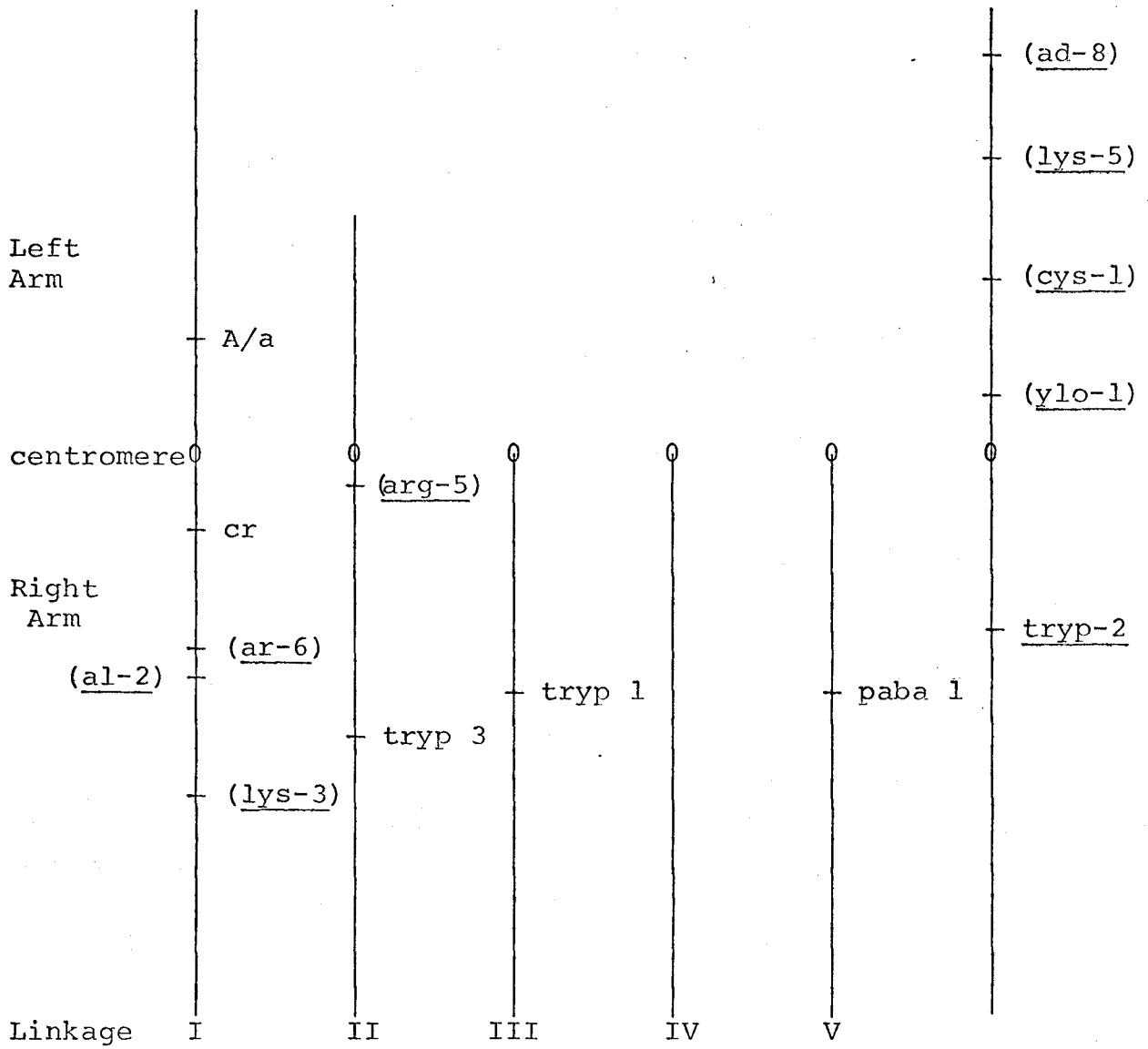
$$\text{map distance} = \frac{\text{total number of recombinants}}{\text{total number of progeny}} \times 100$$

to the data in Table V, ylo-b was calculated to be 0.15 cou distal to aur. As this conclusion was based on a single ascospore isolate it was necessary to obtain greater confidence in crossover frequency estimations through greater numbers of

progeny, but as each of the ascospore isolates reported in the low resolution analysis was manually dissected and individually cultured it was obvious that another more selective technique had to be devised in order to elucidate the fine structure of this region (see Figure (xv)).

Figure (xiv)

The Seven Linkage Groups of Neurospora crassa, Showing Test Marker Sites*




(After Fincham, 1963)

*Markers in brackets used elsewhere in this study.

TABLE IV

Results of Tester Strain Crosses

| Cross | Linkage Group | Germination % | Parental types | Non parental types | $\chi^2$ 3 degrees freedom | Probability of independent segregation |
|---|---|---|---|---|---|---|
| cr ylo-b / + + | I R | 82.3% | cr ylo-b: 87 / + +: 94 | cr +: 31 / + ylo-b: 48 | 42.68 | <.01 |
| A + / a ylo-b | I L | 82.3% | A +: 76 / a ylo-b: 85 | A ylo-b: 57 / a +: 42 | 17.14 | <.01 |
| paba-1 + / + ylo-b | V R | 86.0% | paba-1 +: 16 / + +: 32 | paba-1 ylo-b: 16 / + +: 32 | 5.32 | .15 |
| tryp-1 + / + ylo-b | III R | 85.0% | tryp-1 +: 24 / + ylo-b: 28 | tryp-1 ylo-b: 25 / + +: 21 | 1.02 | .99 |
| tryp-3 + / + ylo-b | II R | 87.5% | tryp-3 ylo-b: 23 / + ylo-b: 16 | tryp-3 ylo-b: 29 / + +: 23 | 3.72 | .29 |

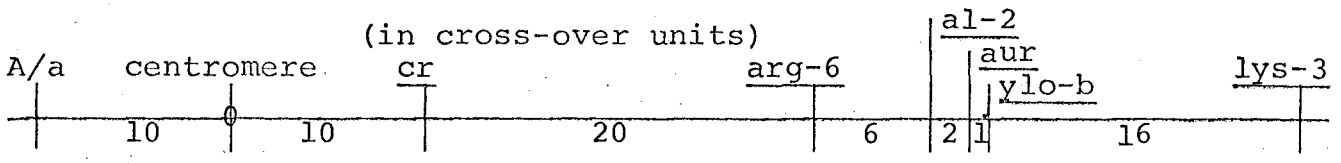
TABLE V

Random Spore Isolate Classification Relating Map Distances in the al Region

| Cross | P.T. | S.C.O.* | D.C.O.** |
|---|---|---|---|
| cr al-2 +<br>+ + ylo-b<br>Germination 70.0% | cr al + (511)***<br>+ + ylo-b 566 | cr al-2 ylo-b (-)<br>+ + + 11<br>cr + ylo-b 207<br>+ al-2 + (226) | cr + + 5<br>+ al-2 ylo-b (-) |
| arg-6 al-2 +<br>+ + ylo-b<br>Germination 82.5% | arg-6 al-2 + (517)<br>+ + ylo-b 522 | arg-6 al-2 ylo-b (-)<br>+ + + 3<br>arg-6 + ylo-b 6<br>+ al-2 + (3) | arg-6 + + 6<br>+ al-2 ylo-b (-) |
| + aur +<br>cr + ylo-b<br>Germination 76.7% | + aur + (444)<br>cr + ylo-b 415 | + + ylo-b 199<br>cr aur + (211)<br>+ aur ylo-b (-)<br>cr + + 1 | + + + 0<br>cr aur ylo-b (-) |

*Postulated "Single Cross Over" class
**Postulated "Double Cross Over" class
***Bracketed scores denote an epistatic situation. Both al-2 and aur were epistatic to ylo-b. The score of the hypostatic class (which was phenotypically indistinguishable from the epistatic class) was noted as (-) eg: cr al-2 + (511) and cr al-2 ylo-b (-). The epistatic score thus was the sum of both classes.

Postulated Map Relationships From Table V



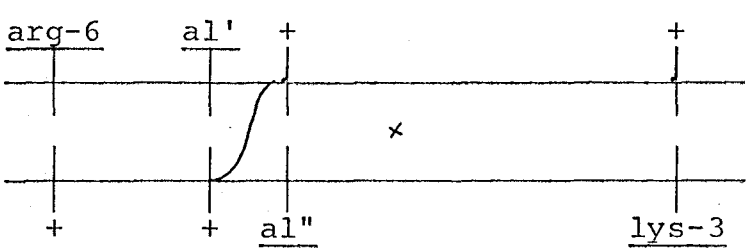

25. High Resolution Studies on the Carotenogenic Mutants of Linkage Group I

Although the principle intent of this section was to order the mutant sites, attention was given to approximating the recombination frequency, especially as such information would serve to place a mutant whose location was beyond the resolution of the selective technique.

To estimate the frequency of recombination between 2 alleles some relationship had to be established between the actual number of viable spores plated and the wt recombinants from FM X FM crosses. The wt prototrophs colonies from cross overs between the pigment markers were readily distinguished from the albino prototroph colonies (see Figure xv) by direct observation of the colonies from plated FM X FM spores.

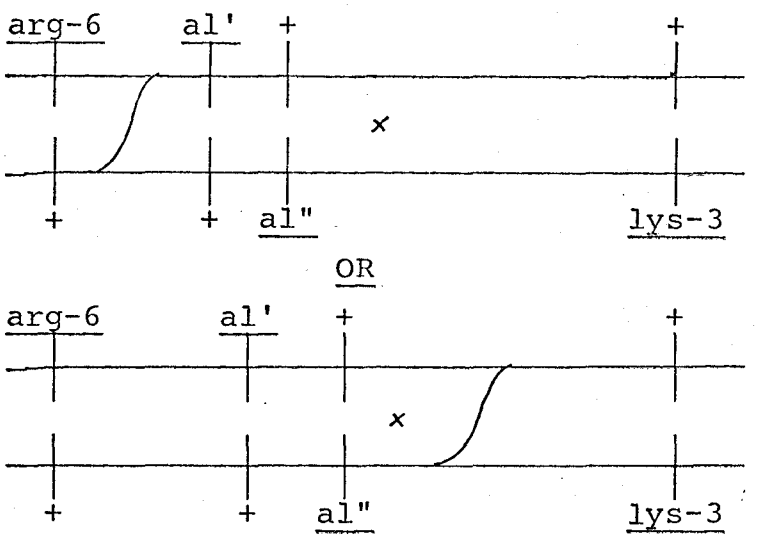
Figure (xv)

wt recombinant




Prototroph Origins

albino
prototrophs




The number of viable spores was originally estimated by plating spores from FM X FM crosses onto complete sorbose medium (containing lysine and arginine). The arginine inhibition effect of lysine uptake (Section 13) resulted in variable germination of the lys-3 strains. Separate tester media duplicated the plating series and was an additional source of experimental error. It was then discovered that viable spore counts could be made directly from the selective (minimal sorbose) medium plates. The colony auxotrophic for arginine had (after 14 days) an arachnoid morphology about 2 mm in diameter in comparison to the very dense 6 mm diameter of a prototrophic colony. The spores auxotrophic for lysine or lysine and arginine produced only a germling hyphal filament which was observable upon microscopic examination and was readily distinguished from the non-germinating spore. Results from such counts indicated the possibility of a statistically acceptable ratio existing between the total number of prototrophs and the total number of viable spores.

If this was so then the numbers of viable spores could be approximated by the total number of prototrophs which could be scored directly from the selective medium plates of the FM X FM wt prototroph hunts.

To test the hypothesis that the number of prototrophs was a reasonable parameter for the approximation of the numbers of viable spores, the following experiment was performed. Three arg-6 and two lys-3 strains were synthesized with the following backgrounds:

| Strain Designate* | Mating Type | Mutation Induced in | 1st Backcross | 2nd Backcross | 3rd Backcross |
|---|---|---|---|---|---|
| arg-6-M | A | L | M | - | - |
| arg-6-OR | a | L | M | ORA | - |
| arg-6-OROR | A | L | M | ORa | ORa |
| lys-3-M | A | L | M | - | |
| lys-3-OR | a | L | M | ORA | |

*for isolation numbers see Materials.
L - Lindegren ORA - Oak Ridge - 74-OR23-1A
M - Mixed ORa - Oak Ridge - 74-OR8-1a

Each of the five strains originated in a Lindegren background and each has been crossed into a mixed background. In addition three strains were crossed to OR A/a. FM strains were synthesized by crossing the arg-6 or lys-3 in the mixed background to a given al strain. To get the desired mating type or to select a suitable morphology the FM strains so derived was backcrossed into OR A/a.

In order to ensure a random sample for the viable spore counts, 4000 spores in selective medium were plated into 25 plates for each cross. Three random plates were taken and sectored. Alternate sectors were observed and scored according to colony phenotype. The statistical analysis (a 3 X 3 contingency test) gives a $\chi^2$ value of 3.51 and with 4 degrees of freedom a probability of 0.51. This would allow that the background effects on intergenic recombination frequencies between arg-6 and lys-3 were not inordinately dispersive, at least with the strains involved.

To relate the frequency of progeny classes obtained from the outside marker strain crosses mentioned in Table VI to those obtained in the high resolution analysis crosses, plates from six crosses of FM X FM strains were scored as shown in Table VII. A correlation of progeny class frequencies was done using the progeny class frequencies from the outside marker strain crosses as the expected frequencies and in each case the $\chi^2$ shows no significant difference. A 6 X 3 contingency table gave a $\chi^2$ of 9.94 with a probability of 0.4 for 10 degrees of freedom. This indicated that the more diverse backgrounds of the carotenoid mutants in the FM strains had only a slightly greater effect on the intergenic recombination frequency between arg-6 and lys-3 than the backgrounds of outside marker strains.

From the expression for calculating a map distance (see Section 24) and the data in Table VI the arg-6- lys-3

TABLE VI

Comparison of Viable Spore and Prototroph Frequencies in Outside Marker Strain Crosses

| Cross | Colony Type: arg-6 lys-3+ | Colony Type: lys-3 or lys-3 arg-6 | arg-6+ lys-3+ | No growth | Germination |
|---|---|---|---|---|---|
| 1. arg-6-MA X lys-3-ORa | 69 | 85 | 15 | 7 | 96.0% |
| 2. arg-6-ORa X lys-3-M-A | 88 | 120 | 32 | 31 | 88.5% |
| 3. arg-6-ORORA X lys-3-OR-a | 37 | 37 | 12 | 15 | 85.0% |
| | | | | Average | 88.8% |
| Totals | 194 | 242 | 59 | 53 | |
| Frequency | .39 | .49 | .12 | .097 | |

*Standard Deviation for Prototroph Frequency 0.065 wtp's
Mean Ratio of Prototrophs to Viable Spores Plated 1:8.4

| Expected: | | | | |
|---|---|---|---|---|
| 1. | 66.23 | 82.62 | 20.14 | 169 |
| 2. | 94.06 | 117.33 | 28.84 | 240 |
| 3. | 33.70 | 42.05 | 10.25 | 86 |
| | 194 | 242 | 59 | 495 |

| (O) Observed | (E) Expected | O-E | $(O-E)^2$ | $\frac{(O-E)^2}{E}$ |
|---|---|---|---|---|
| 69 | 66.23 | 2.77 | 7.67 | 0.11 |
| 88 | 96.06 | 6.06 | 36.72 | 0.39 |
| 37 | 33.70 | 3.30 | 10.89 | 0.32 |
| 85 | 82.62 | 2.38 | 5.66 | 0.07 |
| 120 | 117.33 | 2.67 | 7.13 | 0.06 |
| 37 | 42.05 | 5.05 | 25.50 | 0.60 |
| 15 | 20.14 | 5.14 | 26.42 | 1.31 |
| 32 | 28.84 | 3.16 | 9.98 | 0.35 |
| 12 | 10.25 | 1.75 | 3.06 | 0.30 |
| 495 | 495.22 | | | $\sum = 3.51 \rightarrow \chi^2$ |

D.F. = 4
$\rho = 0.52$

TABLE VI

(continued)

*From the expression:

$$S = \sqrt{\frac{\sum (X_i - M)^2}{N}}$$

where S = standard deviation
Xi = observed value
M = mean of observed values
$N = \sum X_i$

interval was calculated to be 24 cou, a distance which was not inconsistent with the published figures (Huang, 1964). Given this information on map distance and its reliability the number of viable spores can be approximated by the expression:

number of viable spores = 8.4 ± .5 X (total no. of prototrophs).

In order to test the reliability of the wild type prototroph (wt p) frequencies from FM X FM strain crosses a series of duplicate crosses was made and the results statistically analysed for reproducibility (see Table VIII, (a), (b), (c)). The crosses in Table VIII (a) were analysed with a simple 2 X 2 contingency test or with a Yates correction for continuity formula for frequencies too low for the 2 X 2 contingency test. Crosses in Table VIII (b) produced no wt p's on the first cross but from their allele sites as determined from other crosses, wt p's should have been realized. The cross when repeated produced few wt p's and the measure of reproducibility was revealed with a Poisson test. Table VIII (c) crosses are a sample of ten crosses where no wt p's were expected and none were observed therefore no statistical analysis was required. None of the frequencies observed in Table VIII was statistically unacceptable.

TABLE VII

Comparison of Viable Spore and Prototroph Counts in FM X FM Strain Crosses

| Cross | Colony Types | | arg-6$^+$ | $\chi^2$ | p |
|---|---|---|---|---|---|
| | arg-6 lys-3$^+$ | lys-3 or lys-3 arg-6 | lys-3$^+$ | | |
| 15300 arg-6 X JH216 lys-3 | 22 | 27 | 6 | .06 | >0.9 |
| car-1 arg-6 X Y234M471 lys-3 | 19 | 14 | 8 | 4.17 | 0.12 |
| Y256M232 arg-6 X al$^c$ lys-3 | 16 | 18 | 5 | .12 | >0.9 |
| AR 25 arg-6 X 34508 lys-3 | 31 | 39 | 5 | 2.03 | 0.39 |
| Y254M165 arg-6 X RES-25-4y lys-3 | 15 | 7 | 3 | 6.42 | 0.05 |
| 15300 arg-6 X 34508 lys-3 | 12 | 14 | 5 | 1.39 | 0.55 |

$\chi^2$ (from 6 X 3 contingency table) = 9.94

DF = 10

probability = 0.4

TABLE VIII

Analysis of Duplicate Cross Results

(a)

- Analysis based on 2 X 2 contingency test (Hays, 1963).

| Cross | wt prototrophs | total prototrophs | $\chi^2$ | p |
|---|---|---|---|---|
| 34508 *lys-3* | 11 | 297 | | |
| X 15300 *arg-6* | 8 | 477 | 2.98 | 0.09 |
| 15300 *arg-6* | 22 | 278 | | |
| X Y234M471 *lys-3* | 15 | 219 | 0.17 | 0.6 |

- The following analyses were from an expression incorporating the Yates correction for continuity (Hay's, 1963) viz:

$$\chi^2 = \frac{N[(ad - bc) - N/2]^2}{(a + b)(c + d)(a + c)(b + d)}$$

from the standard 2 X 2 contingency table form;

| | | |
|---|---|---|
| a | b | (a + b) |
| c | d | (c + d) |
| (a + c) | (b + d) | N |

| | | | | |
|---|---|---|---|---|
| AR 25 *arg-6* | 2 | 439 | .14 | 0.7 |
| X 34508 *lys-3* | 2 | 384 | | |
| 34508 *arg-6* | 1 | 634 | 1.24 | 0.2 |
| X RES-25-4y *lys-3* | 5 | 690 | | |
| Y254M165 *arg-6* | 4 | 388 | 3.57 | 0.05 |
| X RES-25-4y *lys-3* | 12 | 355 | | |
| $al^c$ *lys-3* | 3 | 251 | 3.01 | 0.05 |
| X 15300 *arg-6* | 10 | 237 | | |
| 46-11-68 *arg-6* | 3 | 116 | .21 | 0.6 |
| X RES-25-4y *lys-3* | 7 | 250 | | |

TABLE VIII (continued)

(b) The following analyses were from a Poisson test according to the expression:

$$f(x) \longrightarrow \frac{\lambda^x e^{-x}}{x!} = p\ (x\lambda)$$

(Kenney, 1954)

The formula applies when the sample (s) is large and the probability ($\theta$) is low. $\lambda$ is the product of s $\theta$. The acceptable limits for the observed score x are presented for the first test results. All scores were found statistically reliable.

| | wt p | total prototrophs | p |
|---|---|---|---|
| ALS-19-S79 arg-6 a<br>X Y234M471 lys-3 A | 0 | 174 | 0.81 |

| f(x) | p |
|---|---|
| f(0) | 0.81 |
| f(1) | 0.16 |
| f(2) | 0.016 |
| f(3) | <0.001 |

| | | | |
|---|---|---|---|
| | 1 | 676 | 0.32 |

| f(x) | p |
|---|---|
| f(0) | 0.45 |
| f(1) | 0.32 |
| f(2) | 0.14 |
| f(3) | <0.01 |

| | | | |
|---|---|---|---|
| car-1 arg-6 a | 0 | 326 | 0.98 |
| X 15300 al-2 lys-3 A | 1 | 2300 | 0.12 |

(c) Neither cross producing wt prototrophs.

| | | |
|---|---|---|
| ALS-19-S79 arg-6 | 0 | 215 |
| X car-1 lys-3 | 0 | 368 |
| Y256M232 arg-6 | 0 | 244 |
| X Y256M220 lys-3 | 0 | 348 |
| Y256M232 arg-6 | 0 | 147 |
| X Y254M165 lys-3 | 0 | 93 |
| ALS-19-S79 arg-6 | 0 | 215 |
| X car-1 lys-3 | 0 | 368 |
| ALS-23-S70 arg-6 | 0 | 111 |
| X Y246M2 lys-3 | 0 | 412 |
| ALS-23-S70 arg-6 | 0 | 432 |
| X Jh216 lys-3 | 0 | 558 |
| RES-25-4y arg-6 | 0 | 302 |
| X JH216 lys-3 | 0 | 145 |
| Y234M471 arg-6 | 0 | 482 |
| X $al^c$ lys-3 | 0 | 366 |
| Y254M165 arg-6 | 0 | 81 |
| X RES-25-4y lys-3 | 0 | 370 |
| Y254M165 arg-6 | 0 | 133 |
| X $al^c$ lys-3 | 0 | 108 |
| Y234M471 arg-6 | 0 | 166 |
| X RES-25-4y lys-3 | 0 | 108 |

To test the selective technique's reliability to produce recombination data from wt p frequencies as opposed to wt's from reversion, contamination or some flaw in the system's logic a series of homoallelic crosses were made with all FM strains. The results of such a series (Table IX) demonstrated no inordinate incidences of wt p frequencies.

These data reveal that possibly one wt p spore in $3.3 \times 10^4$ viable spores may not be accounted for by recombination and probably was due to reversion. Strain RES-25-4y arg-6's tendency to revert will be discussed in a later section.

TABLE IX

FM X FM Homoallelic Crosses

| Cross | wt p | total prototrophs |
|---|---|---|
| 15300 *arg-6* A X 15300 *lys-3* | 0 | 654 |
| ALS-19-S79 *arg-6* A X ALS-19-S79 *lys-3* | 0 | 24 |
| 34508 *arg-6* A X 34508 *lys-3* | 0 | 608 |
| RES-25-4y *arg-6* A X RES-25-4y *lys-3* | 1 | 404 |
| $al^c$ *arg-6* A X $al^c$ *lys-3* | 0 | 138 |
| Y234M471 *arg-6* A X Y234M471 *lys-3* | 0 | 365 |
| Y254M165 *arg-6* a X Y254M165 *lys-3* | 0 | 536 |
| 15300 *arg-6* a X 15300 *lys-3* | 0 | 492 |
| 34508 *arg-6* a X 34508 *lys-3* | 0 | 203 |
| RES-25-4y *arg-6* a X RES-25-4y *lys-3* | 0 | 200 |
| Y234M471 *arg-6* a X Y234M471 *lys-3* | 0 | 365 |
| | 1 | 3989 |

TABLE X

Results of FM X FM Crosses

| arg-6 FM Parent | MT | lys-3 FM Parent | MT | wt p | total prototrophs | viable spores | wt p frequency X $10^{-4}$ | hetero-karyons* (wt) |
|---|---|---|---|---|---|---|---|---|
| AR 25 | A | Y254M165 | a | 1 | 185 | 1554 | 6.4 | 2 |
| AR 25 | A | car-1 | a | 0 | 96 | 806 | 0.0 | 0 |
| AR 25 | A | 15300 | a | 0 | - | 2000 | 0.0 | 0 |
| AR 25 | A | ALS-19-S79 | a | 1 | 260 | 2184 | 4.5 | 0 |
| AR 25 | A | 34508 | a | 2 | 439 | 3687 | 5.4 | 0 |
| AR 25 | A | $al^c$ | a | 2 | 104 | 873 | 22.9 | 1 |
| AR 25 | A | JH216 | a | 3 | 115 | 966 | 31.0 | 2 |
| AR 25 | A | Y234M471 | a | 5 | 141 | 1184 | 42.2 | 0 |
| 15300 | A | Y254M165 | a | 0 | 333 | 2797 | 0.0 | 0 |
| 15300 | A | car-1 | a | 0 | 144 | 1209 | 0.0 | 0 |
| 15300 | A | 15300 | a | 0 | 654 | 5493 | 0.0 | 0 |
| 15300 | A | Y256M220 | a | 0 | 432 | 3628 | 0.0 | 0 |
| 15300 | A | 34508 | a | 5 | 286 | 2402 | 20.8 | 1 |
| 15300 | A | ALS-23-S70 | a | 8 | - | 2000 | 40.0 | 5 |
| 15300 | A | RES-25-4y | a | 4 | 150 | 1260 | 31.7 | 0 |
| 15300 | A | $al^c$ | a | 8 | 488 | 4099 | 19.6 | 5 |
| 15300 | A | Y246M2 | a | 5 | 146 | 1226 | 40.8 | 0 |
| 15300 | A | JH216 | a | 12 | 231 | 1940 | 61.8 | 5 |
| 15300 | A | Y234M471 | a | 17 | 224 | 1881 | 90.3 | 0 |
| Y256M232 | A | Y254M165 | a | 0 | 240 | 2016 | 0.0 | 1 |
| Y256M232 | A | car-1 | a | 0 | 241 | 2024 | 0.0 | 0 |
| Y256M232 | A | 15300 | a | 0 | 466 | 3914 | 0.0 | 0 |
| Y256M232 | A | Y256M220 | a | 0 | 592 | 4972 | 0.0 | 0 |
| Y256M232 | A | ALS-19-S79 | a | 4 | 121 | 1016 | 39.4 | 6 |

| arg-6 FM Parent | MT | lys-3 FM Parent | MT | wt p | total prototrophs | viable spores | wt p frequency X $10^{-4}$ | hetero-karyons* (wt) |
|---|---|---|---|---|---|---|---|---|
| Y256M232 | A | 34508 | a | 5 | 428 | 3595 | 13.9 | 3 |
| Y256M232 | A | RES-25-4y | a | 7 | 105 | 882 | 79.3 | 0 |
| Y256M232 | A | al$^{c}$ | a | 8 | 188 | 1579 | 50.6 | 2 |
| Y256M232 | A | JH216 | a | 11 | 328 | 2755 | 39.9 | 0 |
| Y256M232 | A | Y234M471 | a | 10 | 205 | 1722 | 58.0 | 0 |
| ALS-19-S79 | A | Y254M165 | a | 0 | 182 | 1528 | 0.0 | 12 |
| ALS-19-S79 | A | car-1 | a | 0 | 241 | 2024 | 0.0 | 2 |
| ALS-19-S79 | A | 15300 | a | 0 | 660 | 5544 | 0.0 | 3 |
| ALS-19-S79 | A | Y256M220 | a | 1 | 375 | 3150 | 3.1 | 2 |
| ALS-19-S79 | A | ALS-19-S79 | a | 0 | 24 | 201 | 0.0 | 0 |
| ALS-19-S79 | A | 34508 | a | 6 | 1032 | 8668 | 6.9 | 0 |
| ALS-19-S79 | A | al$^{c}$ | a | 2 | 202 | 1696 | 11.8 | 0 |
| ALS-19-S79 | A | Y246M2 | a | 5 | 419 | 3519 | 14.2 | 0 |
| ALS-19-S79 | A | JH216 | a | 2 | 271 | 2276 | 8.8 | 0 |
| ALS-19-S79 | A | Y234M471 | a | 2 | 316 | 2654 | 7.5 | 0 |
| 34508 | A | Y254M165 | a | 0 | 112 | 940 | 0.0 | 0 |
| 34508 | A | car-1 | a | 0 | 160 | 1344 | 0.0 | 0 |
| 34508 | A | 15300 | a | 0 | 314 | 2637 | 0.0 | 2 |
| 34508 | A | Y256M220 | a | 1 | 477 | 4006 | 2.5 | 3 |
| 34508 | A | 34508 | a | 0 | 608 | 5107 | 0.0 | 0 |
| 34508 | A | RES-25-4y | a | 7 | 1234 | 10365 | 6.7 | 0 |
| 34508 | A | al$^{c}$ | a | 0 | 146 | 1226 | 0.0 | 0 |
| 34508 | A | JH216 | a | 2 | 463 | 3889 | 5.1 | 0 |
| 34508 | A | Y234M471 | a | 0 | 166 | 1394 | 0.0 | 0 |
| ALS-4-S48-64 | A | Y254M165 | a | 0(1?) | 968 | 8131 | 0.0 | 2 |
| ALS-4-S48-64 | A | car-1 | a | 0(1?) | 253 | 2125 | 0.0 | 2 |
| ALS-4-S48-64 | A | Y256M220 | a | 0(1?) | 319 | 2679 | 0.0 | 0 |
| ALS-4-S48-64 | A | ALS-19-S79 | a | 0 | 116 | 974 | 0.0 | 2 |
| ALS-4-S48-64 | A | 34508 | a | 0 | 415 | 3486 | 0.0 | 0 |
| ALS-4-S48-64 | A | al$^{c}$ | a | 4 | 599 | 5031 | 7.9 | 0 |
| ALS-4-S48-64 | A | JH216 | a | 2 | 331 | 2780 | 7.2 | 0 |

| arg-6 FM Parent | MT | lys-3 FM Parent | MT | wt p | total prototrophs | viable spores | wt p frequency X $10^{-4}$ | hetero-karyons* (wt) |
|---|---|---|---|---|---|---|---|---|
| ALS-4-S48-64 | A | Y234M471 | a | 2 | 146 | 1226 | 16.3 | 0 |
| ALS-23-S70 | A | Y254M165 | a | 0 | 73 | 613 | 0.0 | 0 |
| ALS-23-S70 | A | car-1 | a | 0 | 162 | 1360 | 0.0 | 0 |
| ALS-23-S70 | A | Y256M220 | a | 0 | 327 | 2746 | 0.0 | 0 |
| ALS-23-S70 | A | $al^c$ | a | 0 | 281 | 2360 | 0.0 | 1 |
| ALS-23-S70 | A | Y246M2 | a | 0 | 523 | 4393 | 0.0 | 0 |
| ALS-23-S70 | A | JH216 | a | 2 | 432 | 3628 | 5.5 | 0 |
| ALS-23-S70 | A | Y234M471 | a | 2 | 274 | 2301 | 8.6 | 2 |
| RES-25-4y | A | Y254M220 | a | 0 | 451 | 3788 | 0.0 | 4 |
| RES-25-4y | A | car-1 | a | 0 | 197 | 1654 | 0.0 | 0 |
| RES-25-4y | A | 15300 | a | 0 | 854 | 7173 | 0.0 | 2 |
| RES-25-4y | A | ALS-19-S79 | a | 1 | 102 | 856 | 11.6 | 0 |
| RES-25-4y | A | 34508 | a | 0 | 1220 | 10248 | 0.0 | 0 |
| RES-25-4y | A | RES-25-4y | a | 0 | 404 | 3393 | 0.0 | 0 |
| RES-25-4y | A | $al^c$ | a | 2 | 179 | 1503 | 13.3 | 0 |
| RES-25-4y | A | Y246M2 | a | 2 | 58 | 487 | 41.0 | 1 |
| RES-25-4y | A | JH216 | a | 1 | 447 | 3754 | 2.6 | 0 |
| RES-25-4y | A | Y234M471 | a | 3 | 434 | 3645 | 8.2 | 1 |
| $al^c$ | A | Y254M165 | a | 0 | 451 | 3788 | 0.0 | 0 |
| $al^c$ | A | car-1 | a | 0 | 104 | 873 | 0.0 | 1 |
| $al^c$ | A | 15300 | a | 0 | 411 | 3452 | 0.0 | 5 |
| $al^c$ | A | Y256M220 | a | 0 | 187 | 1570 | 0.0 | 0 |
| $al^c$ | A | ALS-19-S79 | a | 4 | 392 | 3292 | 12.1 | 0 |
| $al^c$ | A | 34508 | a | 4 | 256 | 2150 | 18.6 | 1 |
| $al^c$ | A | RES-25-4y | a | 3 (lost) | 820 | 6888 | 4.3 | (lost) |
| $al^c$ | A | $al^c$ | a | 0 | 138 | 1159 | 0.0 | 0 |
| $al^c$ | A | Y246M2 | a | 0 | 258 | 2167 | 0.0 | 0 |
| $al^c$ | A | JH216 | a | 1 | 203 | 1705 | 5.8 | 0 |
| $al^c$ | A | Y234M471 | a | 0 | 15 | 126 | 0.0 | 0 |
| Y234M471 | A | Y254M165 | a | 0 | 249 | 2091 | 0.0 | 1 |

| arg-6 FM Parent | MT | lys-3 FM Parent | MT | wt p | total prototrophs | viable spores | wt p frequency X $10^{-4}$ | hetero-karyons* (wt) |
|---|---|---|---|---|---|---|---|---|
| Y234M471 | A | car-1 | a | 0 | 190 | 1596 | 0.0 | 2 |
| Y234M471 | A | 15300 | a | 0 | 631 | 5300 | 0.0 | 0 |
| Y234M471 | A | Y256M220 | a | 0 | 185 | 1554 | 0.0 | 4 |
| Y234M471 | A | ALS-19-S79 | a | 0 | 76 | 638 | 0.0 | 1? |
| Y234M471 | A | 34508 | a | 0 | 394 | 3309 | 0.0 | 0 |
| Y234M471 | A | $al^c$ | a | 1 | 848 | 7123 | 1.4 | 0 |
| Y234M471 | A | Y246M2 | a | 1 | 38 | 319 | 31.3 | 1 |
| Y234M471 | A | JH216 | a | 0 | 619 | 5199 | 0.0 | 0 |
| Y234M471 | A | Y234M471 | a | 0 | 365 | 3066 | 0.0 | 0 |
| 46-11-68 | A | RES-25-4y | a | 7 | 366 | 3074 | 22.7 | 0 |
| 46-11-68 | A | ALS-25-S72 | a | 3 | 414 | 3477 | 8.6 | 0 |
| ALS-4-S48-64 | A | Y246M2 | a | 3 | 585 | 4914 | 6.1 | 0 |
| ALS-22-S82 | A | 34508 | a | 0 | 932 | 7828 | 0.0 | 0 |
| ALS-22-S82 | A | Y234M471 | a | 0 | 73 | 613 | 0.0 | 0 |
| ALS-22-S82 | A | $al^c$ | a | 0 | 42 | 352 | 0.0 | 0 |
| AR 25 | A | ALS-23-S70 | a | 3 | 314 | 2637 | 11.3 | 0 |
| ALS-23-S70 | A | 15300 | a | 0 | - | 2000 | 0.0 | 0 |
| ALS-19-S79 | A | 34508 | a | 0 | 58 | 487 | 0.0 | 0 |
| ALS-23-S70 | A | 34508 | a | 0 | 523 | 4393 | 0.0 | 0 |
| Y254M165 | a | Y254M165 | A | 0 | 536 | 4502 | 0.0 | 0 |
| Y254M165 | a | Y256M220 | A | 3 | 1875 | 15750 | 1.9 | 3 |
| Y254M165 | a | 15300 | A | 0 | 250 | 2100 | 0.0 | 4 |
| Y254M165 | a | 34508 | A | 8 | 196 | 1646 | 48.6 | 0 |
| Y254M165 | a | ALS-25-S72 | A | 0 | 175 | 1470 | 0.0 | 0 |
| Y254M165 | a | ALS-23-S70 | A | 1 | - | 2000 | 5.0 | 0 |
| Y254M165 | a | RES-25-4y | A | 16 | 743 | 6241 | 25.6 | 0 |
| Y254M165 | a | G2K30 | A | 2 | 420 | 3528 | 5.6 | 0 |
| Y254M165 | a | Y234M471 | A | 14 | 151 | 1268 | 110.4 | 16 |
| car-1 | a | Y254M165 | A | 0 | 42 | 352 | 0.0 | 0 |
| car-1 | a | Y256M220 | A | 0 | 250 | 2100 | 0.0 | 0 |

| arg-6 FM Parent | MT | lys-3 FM Parent | MT | wt p | total prototrophs | viable spores | wt p frequency X $10^{-4}$ | hetero-karyons* (wt) |
|---|---|---|---|---|---|---|---|---|
| car-1 | a | 15300 | A | 1 | 2626 | 22058 | 0.45 | 10 |
| car-1 | a | 34508 | A | 0 | 250 | 2100 | 0.0 | 0 |
| car-1 | a | ALS-25-S72 | A | 0 | - | 2000 | 0.0 | 0 |
| car-1 | a | ALS-23-S70 | A | 1 | 370 | 3108 | 3.2 | 0 |
| car-1 | a | RES-25-4y | A | 5 | 266 | 2234 | 22.3 | 0 |
| car-1 | a | G2K30 | A | 2 | 411 | 3452 | 57.9 | 0 |
| car-1 | a | Y234M471 | A | 9 | 258 | 2167 | 41.5 | 0 |
| 15300 | a | Y254M165 | A | 0 | - | 4000 | 0.0 | 0 |
| 15300 | a | Y256M220 | A | 0 | 342 | 2872 | 0.0 | 0 |
| 15300 | a | 15300 | A | 0 | 492 | 4132 | 0.0 | 0 |
| 15300 | a | 34508 | A | 8 | 477 | 4006 | 19.9 | 0 |
| 15300 | a | ALS-25-S72 | A | 5 | - | 2000 | 25.0 | 1 |
| 15300 | a | ALS-23-S70 | A | 4 | 311 | 2612 | 15.3 | 0 |
| 15300 | a | RES-25-4y | A | 4 | 361 | 3032 | 13.19 | 1 |
| 15300 | a | G2K30 | A | 1 | 497 | 4174 | 2.39 | 0 |
| 15300 | a | Y234M471 | A | 20 | 278 | 2335 | 86.0 | 0 |
| ALS-19-S79 | a | Y254M165 | A | 0 | 360 | 3024 | 0.0 | 0 |
| ALS-19-S79 | a | Y256M220 | A | 0 | 375 | 3150 | 0.0 | 2 |
| ALS-19-S79 | a | 15300 | A | 1 | - | 2000 | 5.0 | 7 |
| ALS-19-S79 | a | 34508 | A | 2 | 2011 | 16892 | 1.1 | 0 |
| ALS-19-S79 | a | ALS-25-S72 | A | 0 | - | 4000 | 0.0 | 0 |
| ALS-19-S79 | a | RES-25-4y | A | 1 | 279 | 2343 | 4.2 | 0 |
| ALS-19-S79 | a | G2K30 | A | 2 | 168 | 1411 | 14.1 | 1 |
| ALS-19-S79 | a | Y234M471 | A | 1 | 676 | 5678 | 1.7 | 0 |
| ALS-22-S82 | a | Y254M165 | A | 0 | - | 2000 | 0.0 | 0 |
| ALS-22-S82 | a | Y256M220 | A | 0 | 770 | 6468 | 0.0 | 4 |
| ALS-22-S82 | a | 15300 | A | 0 | 314 | 2637 | 0.0 | 4 |
| ALS-22-S82 | a | 34508 | A | 0 | 660 | 5544 | 0.0 | 0 |
| ALS-22-S82 | a | ALS-25-S72 | A | 0 | - | 4000 | 0.0 | 0 |
| ALS-22-S82 | a | RES-25-4y | A | 0 | - | 4000 | 0.0 | 0 |

| arg-6 FM Parent | MT | lys-3 FM Parent | MT | wt p | total prototrophs | viable spores | wt p frequency X $10^{-4}$ | hetero-karyons* (wt) |
|---|---|---|---|---|---|---|---|---|
| ALS-22-S82 | a | Y234M471 | A | 0 | 284 | 2385 | 0.0 | 0 |
| 34508 | a | Y254M165 | A | 0 | 421 | 3536 | 0.0 | 0 |
| 34508 | a | Y256M220 | A | 0 | 331 | 2780 | 0.0 | >30 |
| 34508 | a | 15300 | A | 0 | 364 | 3057 | 0.0 | 0 |
| 34508 | a | 34508 | A | 0 | 203 | 1705 | 0.0 | 0 |
| 34508 | a | ALS-25-S72 | A | 0 | - | 2000 | 0.0 | 0 |
| 34508 | a | ALS-23-S70 | A | 1 | 210 | 1764 | 5.6 | 0 |
| 34508 | a | RES-25-4y | A | 7 | 412 | 3460 | 20.2 | 0 |
| 34508 | a | G2K30 | A | 3 | 374 | 3141 | 9.5 | 0 |
| 34508 | a | Y234M471 | A | 1 | 271 | 2276 | 4.4 | 0 |
| RES-25-4y | a | Y254M165 | A | 0 | 1186 | 9962 | 0.0 | 0 |
| RES-25-4y | a | Y256M220 | A | 0 | 309 | 2595 | 0.0 | 2 |
| RES-25-4y | a | 15300 | A | 0 | 252 | 2116 | 0.0 | 0 |
| RES-25-4y | a | 34508 | A | 0 | 279 | 2343 | 0.0 | 0 |
| RES-25-4y | a | ALS-25-S72 | A | 0 | - | 2000 | 0.0 | 0 |
| RES-25-4y | a | ALS-23-S70 | A | 0 | - | 2000 | 0.0 | 0 |
| RES-25-4y | a | RES-25-4y | A | 1 | 222 | 1864 | * | 0 |
| RES-25-4y | a | G2K30 | A | 1 | 480 | 4032 | 2.4 | 0 |
| RES-25-4y | a | Y234M471 | A | 3 | 787 | 6610 | 4.5 | 0 |
| $al^c$ | a | Y254M165 | A | 0 | 279 | 2343 | 0.0 | 0 |
| $al^c$ | a | Y256M220 | A | 0 | 77 | 646 | 0.0 | 0 |
| $al^c$ | a | 15300 | A | 5 | - | 2000 | 25.0 | 0 |
| $al^c$ | a | ALS-25-S72 | A | 0 | 61 | 512 | 0.0 | 0 |
| $al^c$ | a | RES-25-4y | A | 1 | 53 | 445 | 22.4 | 0 |
| $al^c$ | a | Y234M471 | A | 12 | 1320 | 11088 | 10.8 | 0 |
| $al^c$ | a | 34508 | A | 1 | 342 | 2872 | 3.5 | 0 |
| 80-96 | a | Y254M165 | A | 0 | 215 | 1806 | 0.0 | 0 |
| 80-96 | a | Y256M220 | A | 1 | 280 | 2352 | 4.2 | 4 |

* Irregular morphology

| arg-6 FM Parent | MT | lvs-3 FM Parent | MT | wt p | total prototrophs | viable spores | wt p frequency X $10^{-4}$ | hetero-karyons * (wt) |
|---|---|---|---|---|---|---|---|---|
| 80-96 | a | 15300 | A | 0 | - | 2000 | 0.0 | 5 |
| 80-96 | a | 34508 | A | 0 | - | 2000 | 0.0 | 0 |
| 80-96 | a | ALS-25-S72 | A | 0 | - | 2000 | 0.0 | 0 |
| 80-96 | a | ALS-23-S70 | A | 1 | - | 2000 | 5.0 | 0 |
| 80-96 | a | RES-25-4y | A | 2 | - | 2000 | 10.0 | 1 |
| 80-96 | a | G2K30 | A | 0 | - | 2000 | 0.0 | 0 |
| 80-96 | a | Y234M471 | A | 0 | - | 2000 | 0.0 | 2 |
| JH216 | a | Y256M220 | A | 0 | 201 | 1688 | 0.0 | 1 |
| JH216 | a | 15300 | A | 0 | 84 | 705 | 0.0 | 1 |
| JH216 | a | 34508 | A | 0 | 87 | 730 | 0.0 | 0 |
| JH216 | a | ALS-25-S72 | A | 0 | 177 | 1486 | 0.0 | 0 |
| JH216 | a | ALS-23-S70 | A | 0 | 339 | 2847 | 0.0 | 0 |
| JH216 | a | Y234M471 | A | 0 | 178 | 1495 | 0.0 | 0 |
| Y234M471 | a | Y254M165 | A | 0 | 212 | 1780 | 0.0 | 0 |
| Y234M471 | a | Y256M220 | A | 0 | 185 | 1554 | 0.0 | 0 |
| Y234M471 | a | 15300 | A | 0 | 492 | 4132 | 0.0 | 0 |
| Y234M471 | a | 34508 | A | 0 | 360 | 3024 | 0.0 | 0 |
| Y234M471 | a | ALS-25-S72 | A | 0 | - | 2000 | 0.0 | 0 |
| Y234M471 | a | ALS-23-S70 | A | 0 | 180 | 1512 | 0.0 | 0 |
| Y234M471 | a | RES-25-4y | A | 2 | 274 | 2301 | 8.7 | 0 |
| Y234M471 | a | G2K30 | A | 0 | 156 | 1310 | 0.0 | 0 |
| Y234M471 | a | Y234M471 | A | 0 | 356 | 2990 | 0.0 | 0 |
| 46-11-68 | a | RES-25-4y | A | 6 | 137 | 1150 | 52.1 | 0 |

*From conidial isolate data of wt p and apparent wt p's.

The complete results from all linkage group I high resolution FM X FM crosses appears in Table X. This table is a composite containing all the data for Phases I - IV plus the report on complementing heterokaryons detected.

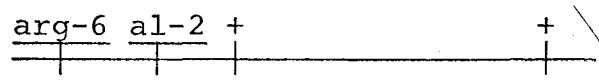
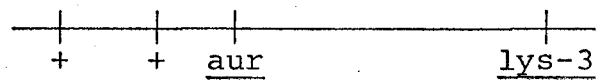
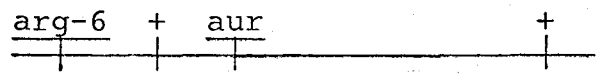
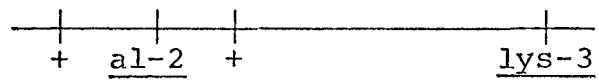
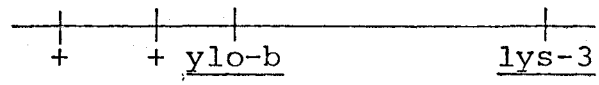
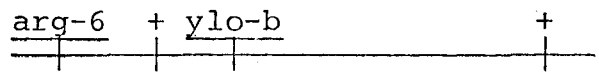
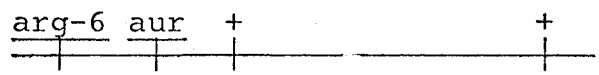
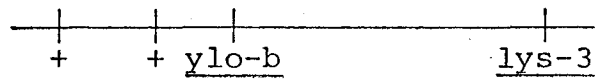
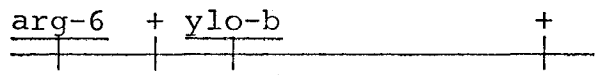
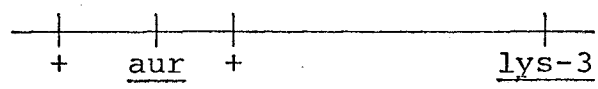
Where the total prototrophs and viable spore frequencies were scored "- 2000" or "- 4000", in Table X, this indicated that the precise number of prototrophs was not scorable. This was due: 1) to a propensity for some strains to sorbose escape (ALS-25-S72, G2K30) which is an event that could be expected in the nuclear population of 14 day old colonies, 2) reversion of either of the two FM markers (lys-3$^-$ → lys-3$^+$ in RES-25-4y lys-3 A). Strains G2K30 and 46-11-68 both possessed wispy growth, impaired fertility and complementability. The correlation between fertility and complementation has been previously noted in yeast (Dorfman, 1964).

A variety of new phenotypes also were observed. These included: 1) two "clock" phenotypes (Sussman, Durkee and Lowry, 1962), 2) several sorbose resistant strains (Klingmüller, 1967), 3) a very pale yellow (not related to the al region), and 4) several intermediate type colonial phenotype strains.

Phase I purported to correlate the marker order established through low resolution analysis (manually dissected spores, see Section 24) to that observed using the high resolution selective technique. Map distances were not provided in Table XI as it was proof of order that was of concern.

TABLE XI

Phase I - Ordering al-2, aur ylo-b

| Cross | wt p/total prototrophs | Probable Map Relationships |
|---|---|---|
| al-2 arg-6 A<br>X aur lys-3 a | 5/286 |  |
| al-2 arg-6 a<br>X aur lys-3 A | 8/477 |  |
| al-2 lys-3 A<br>X aur arg-6 a | 0/364 |  |
| al-2 lys-3 a<br>X aur arg-6 A | 0/314 |  |
| al-2 arg-6 A<br>X ylo-b lys-3 a | 4/150 |  |
| al-2 arg-6 a<br>X ylo-b lys-3 A | 4/361 |  |
| al-2 lys-3 A<br>X ylo-b arg-6 a | 0/854 |  |
| al-2 lys-3 a<br>X ylo-b arg-6 A | 0/252 |  |
| aur arg-6 A<br>X ylo-b lys-3 a | 7/1234 |  |
| aur arg-6 a<br>X ylo-b lys-3 A | 7/412 |  |
| aur lys-3 A<br>X ylo-b arg-6 a | 0/279 |  |
| aur lys-3 a<br>X ylo-b arg-6 A | 0/1220 |  |

The order of al-2 aur and ylo-b mutant sites determined with the selective technique corresponded to the tentative low resolution map obtained in Section 24, Figure (xv). If the total number of viable spores was estimated, the map distance approximations could be interpolated for the aur - ylo-b interval as follows:

$$\text{map distance approximations} = \frac{\text{wt p X 2}}{\text{total prototroph X 8.4}}{}^{*}\text{X 100}$$

$$= \frac{14 \text{ X } 2 \text{ X } 100}{1646 \text{ X } 8.4}$$

$$= 0.20$$

*a factor for converting total prototrophs into total viable spores calculated earlier in this section.

The 0.20 cou high resolution map distance approximation correlates well with the 0.15 cou value from the low resolution analysis.

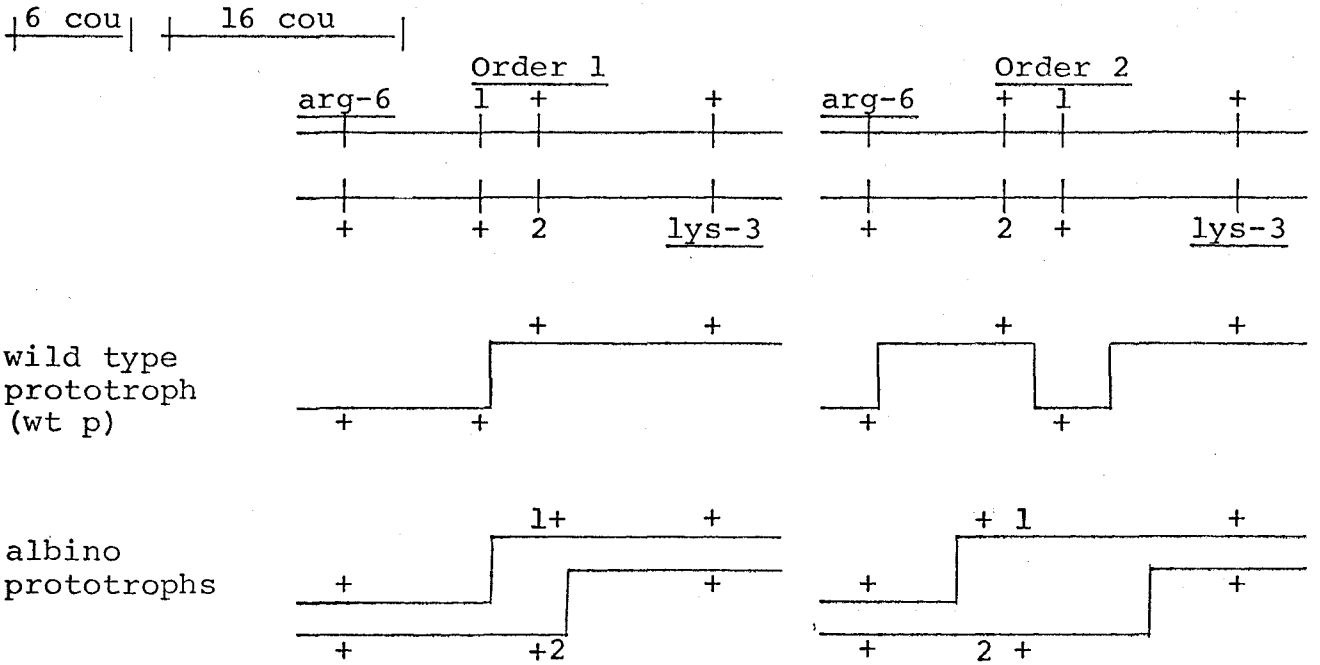
Phase II correlates the order of mutant sites previously reported by other authors for selected albino strains, with the orders obtained using the selective technique. The rationale for ordering carotenoidless mutants is explained with reference to Figure (xvi).

It could be inferred that if wt p's appeared in Cross Type 1 and not in Cross Type 2 that Order 1 is implied. If no wt p's appeared in either Cross Type 1 or 2 fhen the mutant sites were close together. If wt p's appeared in both Cross Types 1 and 2 the ratio was the deciding factor. High negative interference (Pritchard, 1955; Chase and

Figure (xvi)

Ordering Rationale

Cross Type 1

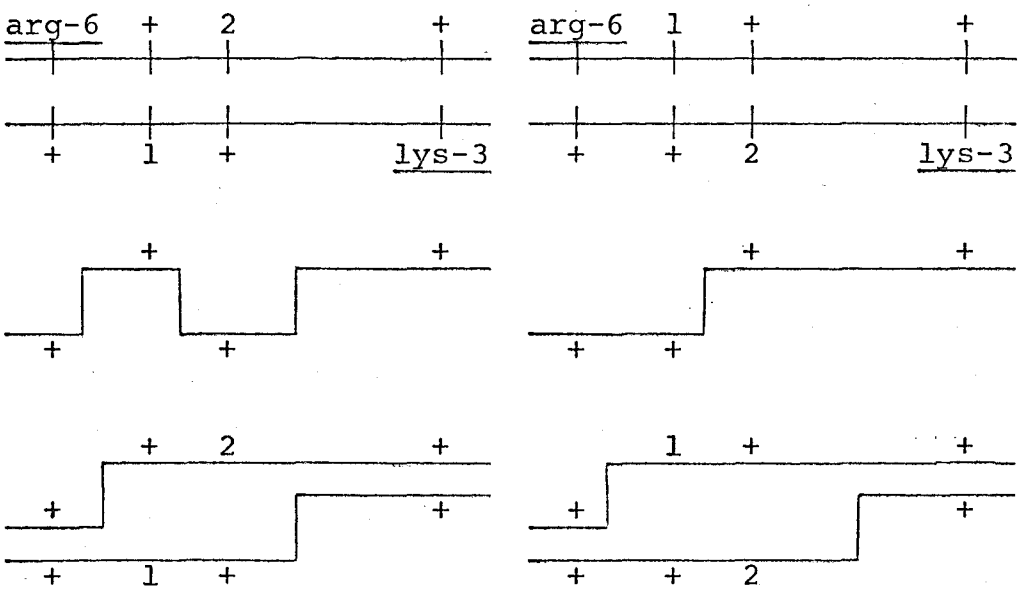



| | Order 1 | Order 2 |
|---|---|---|
| wt p ratio expected | 400x | x |
| Recombination frequency approximation | $\frac{400x}{8.4^{*} \times \text{total prototrophs}}$ | $\frac{x}{8.4 \times \text{total prototrophs}}$ |

*8.4 is the factor converting total prototrophs to approximate number of viable spores plated.

Figure (xvi) (continued)

Cross Type 2

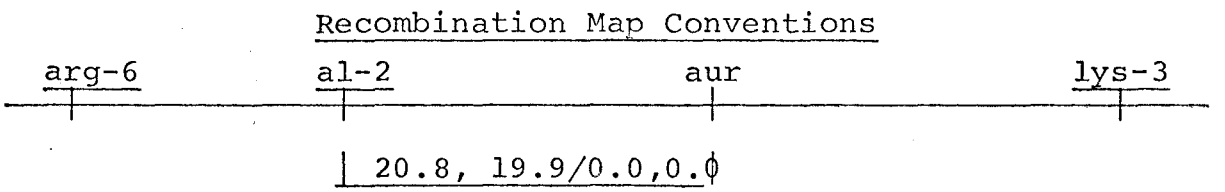



| | | |
|---|---|---|
| wt p ratio expected | x | 400x |
| Recombination frequency approximation | $\frac{x}{8.4 \text{ X total prototrophs}}$ | $\frac{400x}{8.4 \text{ X total prototrophs}}$ |

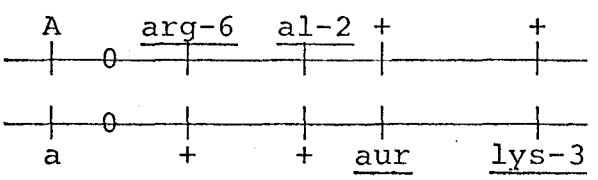
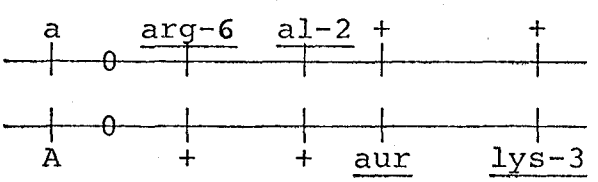
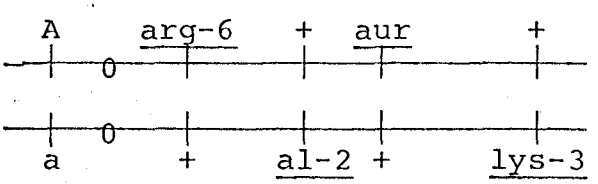
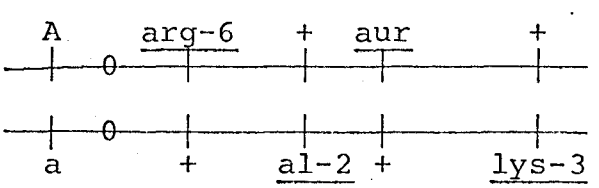
Doermann, 1958; Sherman and Roman, 1963) may have effectively increased the probability of triple cross-overs (but not 400 fold). Reversion on the interallelic recombination mechanism, i.e. conversion may also have effected the frequency of the triple cross-over class.

A convention was invoked to depict the cross-over types, i.e.

Figure (xvii)




This form summarizes the following crosses:

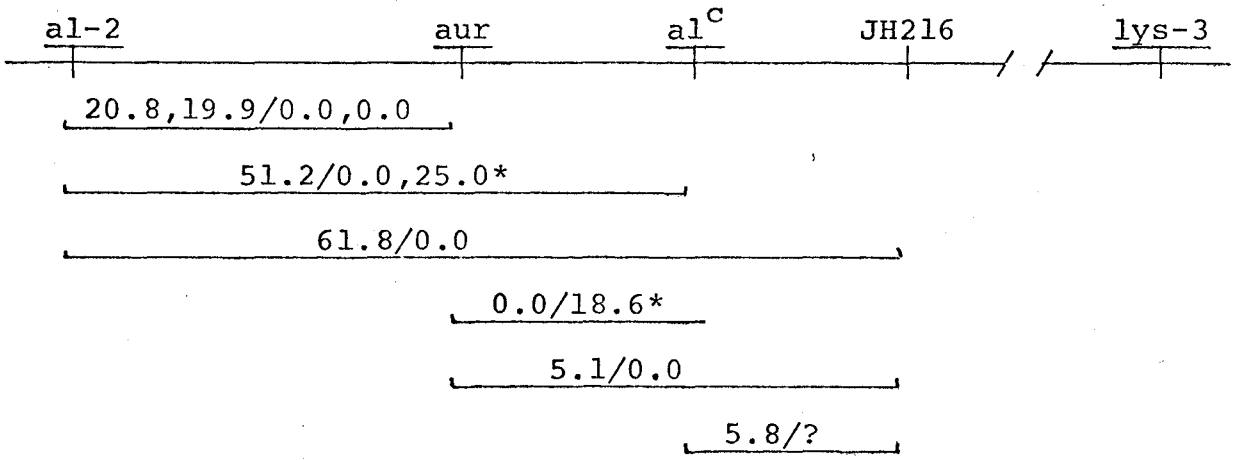
| | wt p | al prototrophs | wt p frequency X $10^{-4}$ |
|---|---|---|---|
|  | 5 | 286 | 20.8 |
|  | 8 | 477 | 19.9 |
|  | 0 | 314 | 0.0 |
|  | 0 | 364 | 0.0 |

The recombination or more correctly the mutant site order map presents the order concluded by the author. The scores before the "/" represent Cross Type 1 in both mating type configurations, e.g. crosses (a) and (b). Scores after the "/" represent Cross Type 2 in both mating type configurations, e.g. crosses (c) and (d).

Figure (xviii)

Phase II - FM X FM Crosses Testing Previous Orders

Postulated map relations from previous authors




*The inconsistencies with $al^c$ crosses appear throughout this study. In the original crosses to arg-6 and lys-3 to obtain FM strains the "crisp" phenotype occurred along with a host of other morphological aberrations. Contamination was unlikely as $al^c$ X $al^c$ crosses produce only albino progeny. Some chromosomal anomally like an inversion duplication is suspected and will be discussed further in a later section. It ($al^c$) was an unfortunate choice of marker.

Exclusive of $al^c$ the wt p frequencies reported in Figure (xviii) confirm the postulated map relations from previous authors.

Phase III invoked the same conventions as Figure (xviii) to record the order of markers for which both FM strains were available for Cross Type 1 and Cross Type 2. The results of these "transposition cross sets" are given in Figure (xix).

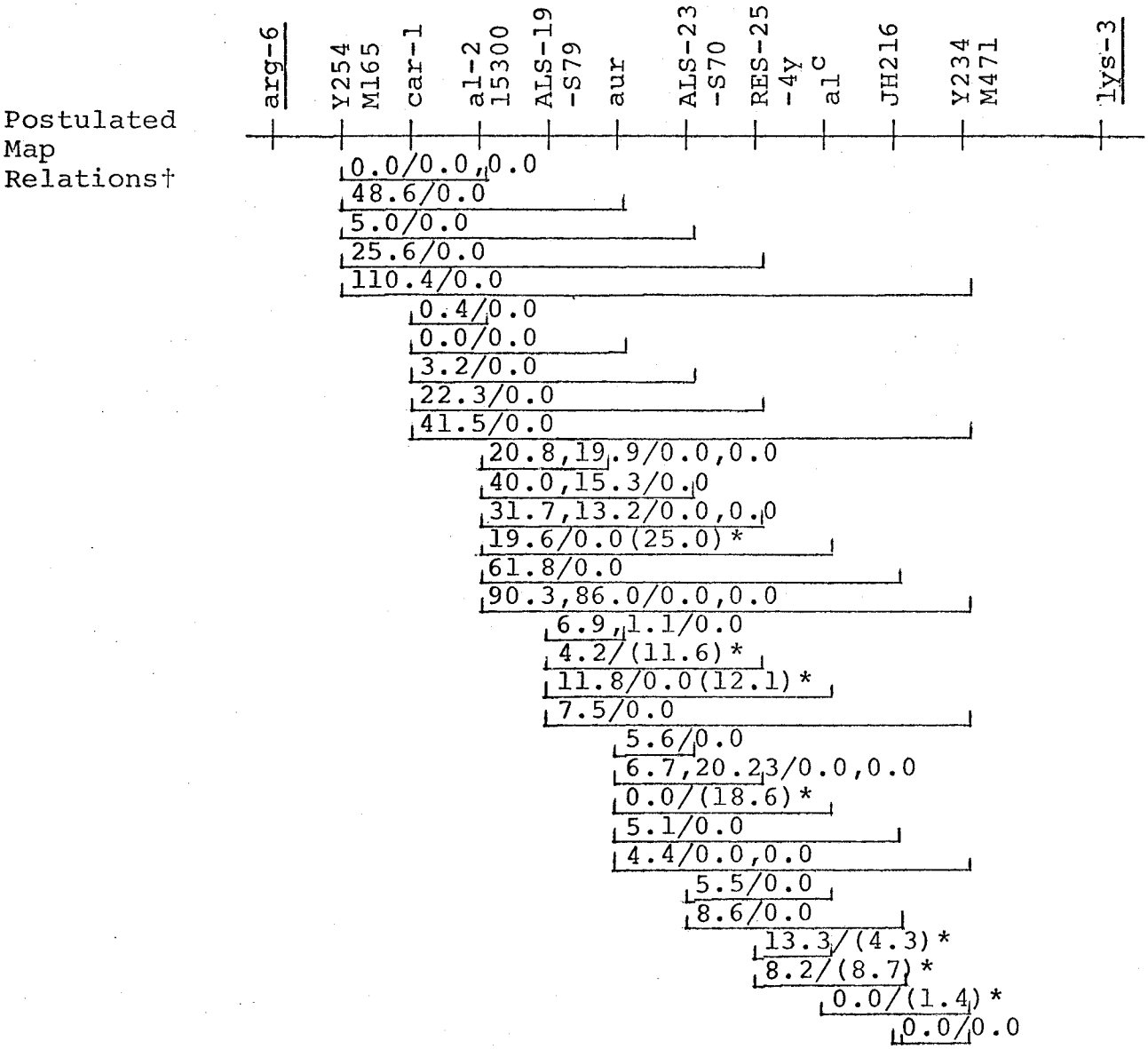
Phase III transposition cross set results revealed several interesting features of the developing recombination map: 1) car-1 had a conspicuously lower wt p frequency than neighbouring Y254M165 or 15300 al-2. This may have been due to $rec^+$ genes affecting interallelic recombination not unlike the rec II type genes that affected interallelic recombination in hist-1 (Jessop and Catcheside, 1965).

2) Additional evidence for rec genes or background effects were seen in the different wt p frequencies from 15300 al-2 A and 15300 al-2 a when crossed to common strains. The different backgrounds arose when 15300 al-2 arg-6 a was backcrossed into OR-74-1A to obtain the opposite mating type strain viz 15300 al-2 arg-6 A.

| Common lys-3 parent | 15300 al-2 arg-6 a wt p frequency X $10^{-4}$ | 15300 al-2 arg-6 A wt p frequency X $10^{-4}$ |
|---|---|---|
| 34508 lys-3 A/a | 19.9 | 20.8 |
| ALS-23-S70 lys-3 A/a | 15.3 | 40.0 |
| RES-25-4y lys-3 A/a | 13.2 | 31.7 |
| Y234M471 lys-3 A/a | 86.0 | 90.3 |

Figure (xix)

Phase III - Transposition Cross Sets




†The interval between alleles is constant for graphic purposes only.

*Bracketed frequencies indicate contradictions to the postulated order.

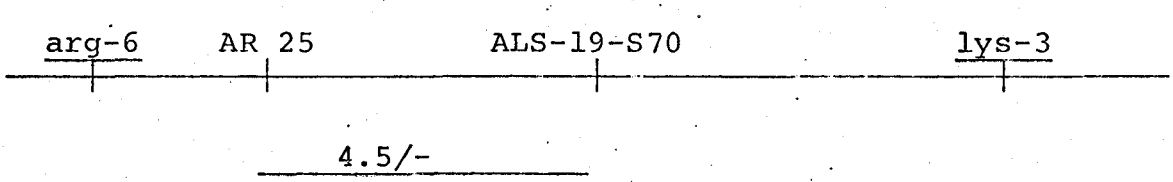
3) An additive map was obviously impossible as map distance approximations showed considerable fluctuation, in some cases a tenfold difference for similar distances, e.g.

Y254M165 arg-6 a X 34508 lys-3 48.6 wt p X $10^{-4}$

Y254M165 arg-6 a X ALS-23-S70 lys-3 5.0 wt p X $10^{-4}$

4) $al^{c}$ accounted for more than half the order contradictions.

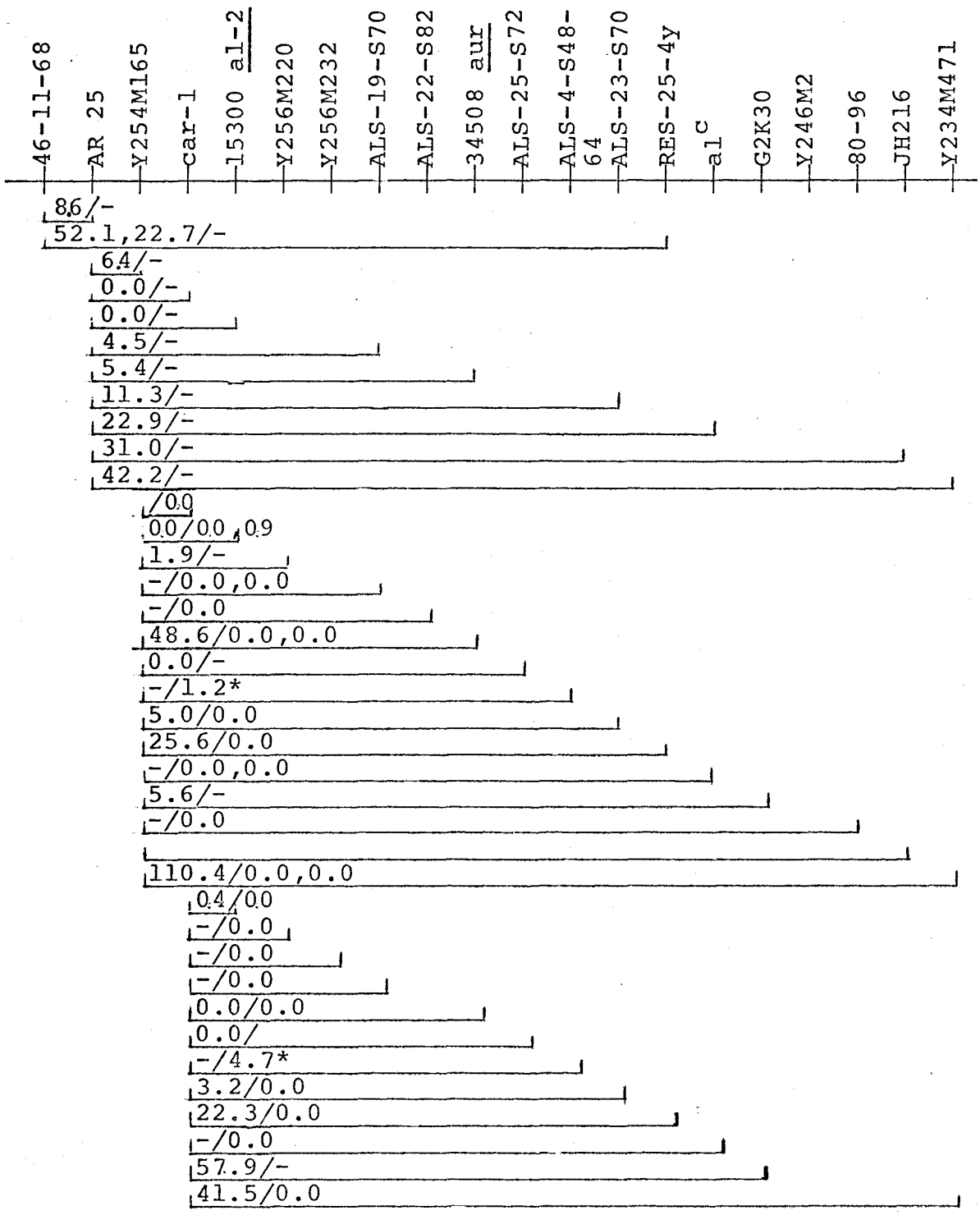
Ancillary strains synthesized with only one FM configuration are integrated with the Phase III results and presented in Figure (xx). Where the sign "-" appears after the "/" sign for a cross set this means that the FM strain was not successfully synthesized e.g.

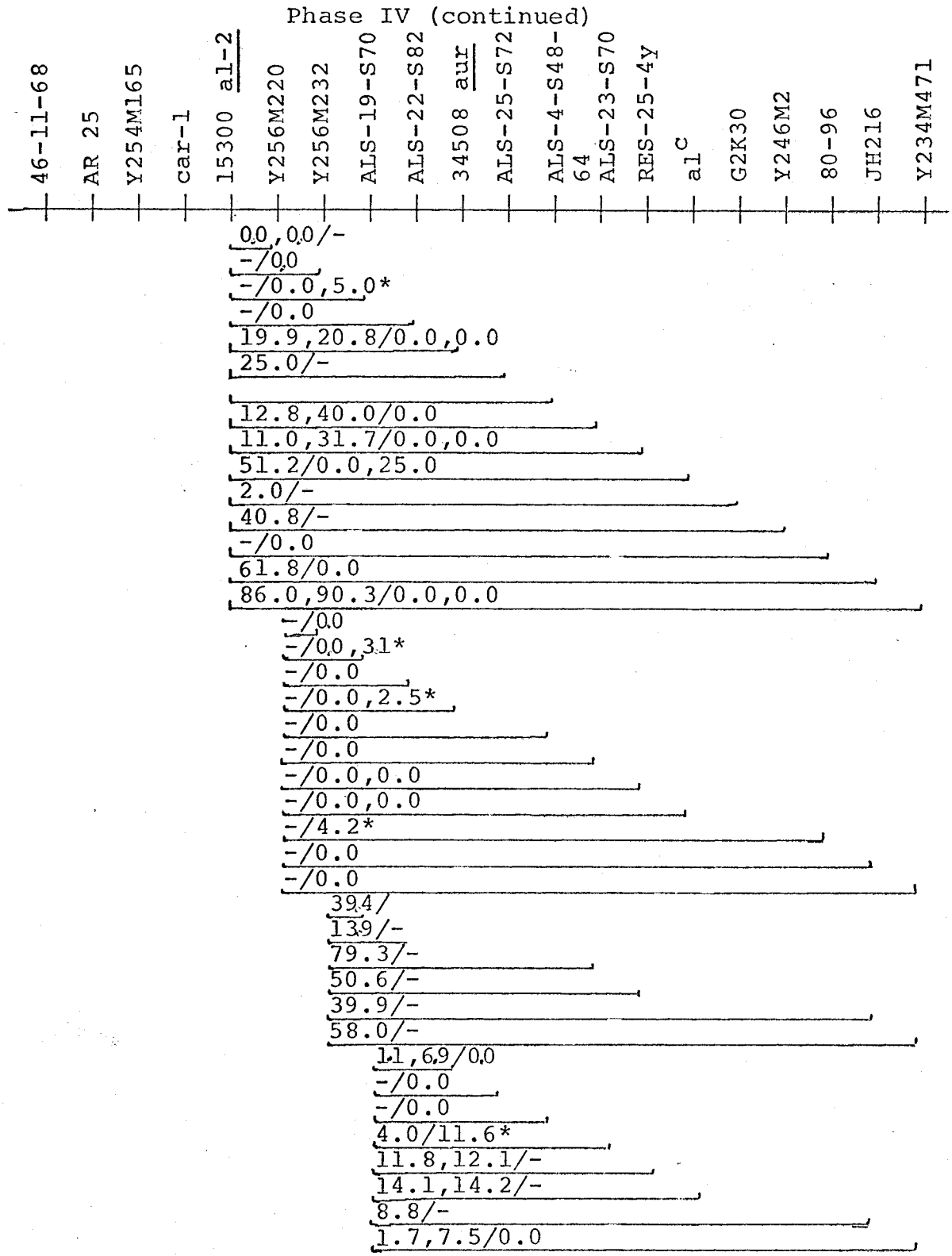



In the illustrated case AR 25 lys-3 was not available so the transpositioned marker configuration cross results were not obtained. Ordering is based entirely on cross results of AR 25 arg-6. Phase IV (Figure (xx)) reports all results in Table X except the homoallele crosses which appeared in Table IX.

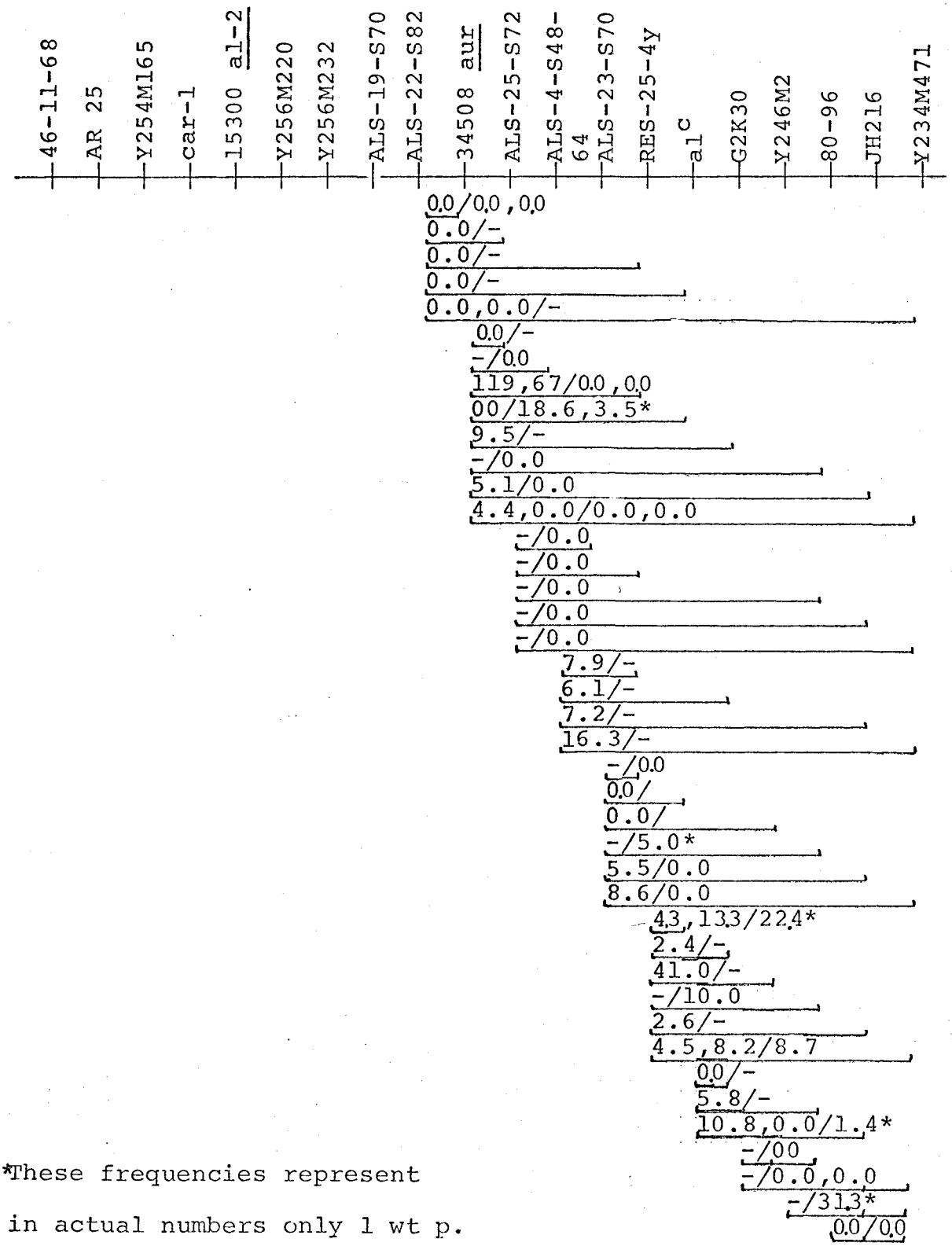
Figure (xx)

Phase IV - Complete FM X FM Cross Results




Phase IV (continued)
46-11-68
AR 25
Y254M165
car-1
15300 a1-2
Y256M220
Y256M232
ALS-19-S70
ALS-22-S82
34508 aur
ALS-25-S72
ALS-4-S48-64
ALS-23-S70
RES-25-4y
a1c
G2K30
Y246M2
80-96
JH216
Y234M471
0.0,0.0/-
-/0.0
-/0.0,5.0*
-/0.0
19.9,20.8/0.0,0.0
25.0/-
12.8,40.0/0.0
11.0,31.7/0.0,0.0
51.2/0.0,25.0
2.0/-
40.8/-
-/0.0
61.8/0.0
86.0,90.3/0.0,0.0
-/0.0
-/0.0,3.1*
-/0.0
-/0.0,2.5*
-/0.0
-/0.0
-/0.0,0.0
-/0.0,0.0
-/4.2*
-/0.0
-/0.0
39.4/
13.9/-
79.3/-
50.6/-
39.9/-
58.0/-
1.1,6.9/0.0
-/0.0
-/0.0
4.0/11.6*
11.8,12.1/-
14.1,14.2/-
8.8/-
1.7,7.5/0.0

Phase IV (continued)

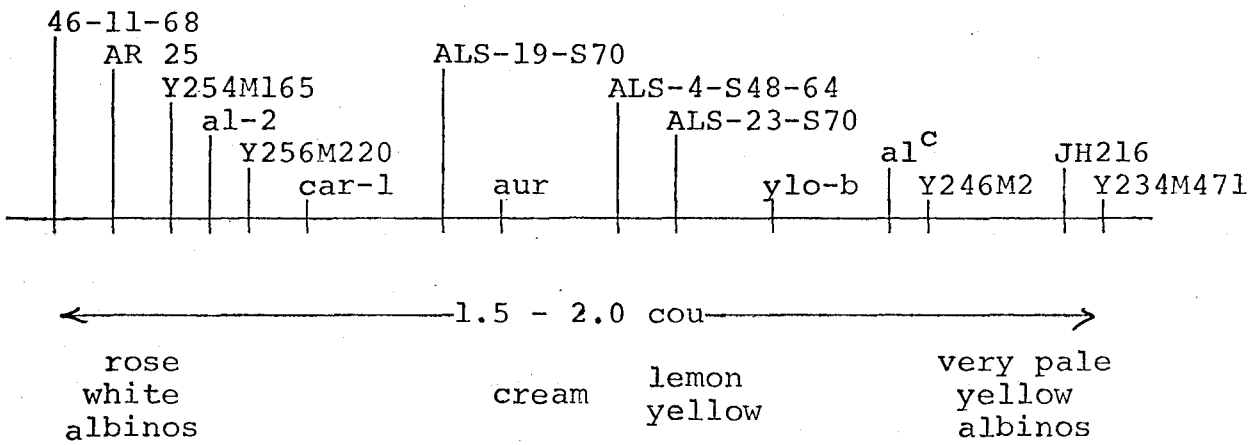



*These frequencies represent in actual numbers only 1 wt p.

Phase IV results indicate four general features of the region. The first is that the mutants expressing the same phenotype seem to be closely associated on the recombination map (see Figure (xxi)).

Figure (xxi)

Hypothetical Map Relations from Map Distance Approximations




The second is that if the map distance approximations were analyzed, the length of the region under study was 1.5 - 2.0 cou in length. This figure was based on estimations from the highest observed wt p frequencies viz

Y254M165 arg-6 X Y234M471 lys-3

15300 al-2 arg-6 X Y234M471 lys-3

AR 25 arg-6 X Y234M471 lys-3

Y256M232 arg-6 X Y234M471 lys-3

Thirdly, the greatest number of contradictory wt p incidences (73%) seem to involve only a single wt p colony. The last general observation is that in some cases the selective technique indicates a certain order but the map distance

approximations indicate another. Furthermore, often times the order is established by a single wt p which it could be argued arose from a triple cross-over a revertant or a conversion product. In such cases the order established by the selective technique is the one reported but it was noted that if map approximations were considered: car-1 would be located adjacent and proximal to ALS-19-S70 and AR 25 would be located adjacent and distal to Y256M232.

Several specific observations require note: 1) ALS-22-S82 produced no wt p's with any cross attempted. When the ALS-22-S82 arg-6 was being synthesized >3000 spores were dissected before the FM strain was isolated. The ALS-22-S82 lys-3 strain was synthesized but was completely sterile. ALS-22-S82 is possibly a deletion mutant for a region distal to ALS-19-S70 which caused chromatid pairing problems hindering cross over formation. 2) ALS-25-S72 arg-6 produced spreading colonies on sorbose plates that displayed many of the characteristics of chronic sorbose escape (Klingmuller, , 1967a,b,c; Crocken and Tatum, 1967). The colonies so formed tended to inhibit each other's growth impairing conidiation and recognition of the colonies' carotenoid phenotype. The mutant site was allocated to a region adjacent to aur 34508 on the basis of phenotype, the order and map distance approximation from one successful cross to 15300 al-2, 3) G2K30 lys-3 possessed very poor growth which made carotenoid phenotype determination difficult for some crosses. G2K30 also exhibited

some features of the sorbose resistance or the sorbose escape syndrome. 4) Y256M232 arg-6 had excellent growth and a high frequency of recombination with successful crosses. Sterility was a problem with most crosses with this strain. Three independent Y256M232 lys-3 strains were isolated that when crossed produced few or no spores.

26. Complementation

As a preliminary screening device all of the carotenoid mutants with the "A" mating type were paired using the mixed culture technique (Section 19). Strains previously with the "a" mating type were crossed into OR-74-1A as this strain possessed the most desirable heterokaryon incompatibility loci combination for this study (Neurospora Newsletter #2: 24). Each pairwise combination was duplicated for each of two screenings, a total of four trials for each pair. The results were reproducible and were presented in Figure (xxii).

Strains of the "U" group (see Figure (xxii)) failed to complement significantly with any other marker. Group I markers displayed a general tendency to complement with both albinos and yellows in Group II. Some strains like $al^{c}$ aur (Group II) and $al^{s}$ (Group I) showed only intermittent instances of complementation. Strain E54R11 gave evidence of extensive complementation but was morphologically similar to the "crisp" (see Discussion) strains with some yellow pigment in the terminal conidia of mature colonies. The additional feature of approximately a 10% hyaline spore frequency discouraged further study of strain

E54R11. Of great interest was the complementation between Y234M471 and 80-96 as this represented the first example of interallelic complementation, if Hungate's evaluation of the region as two discrete loci was correct (Hungate, 1945).

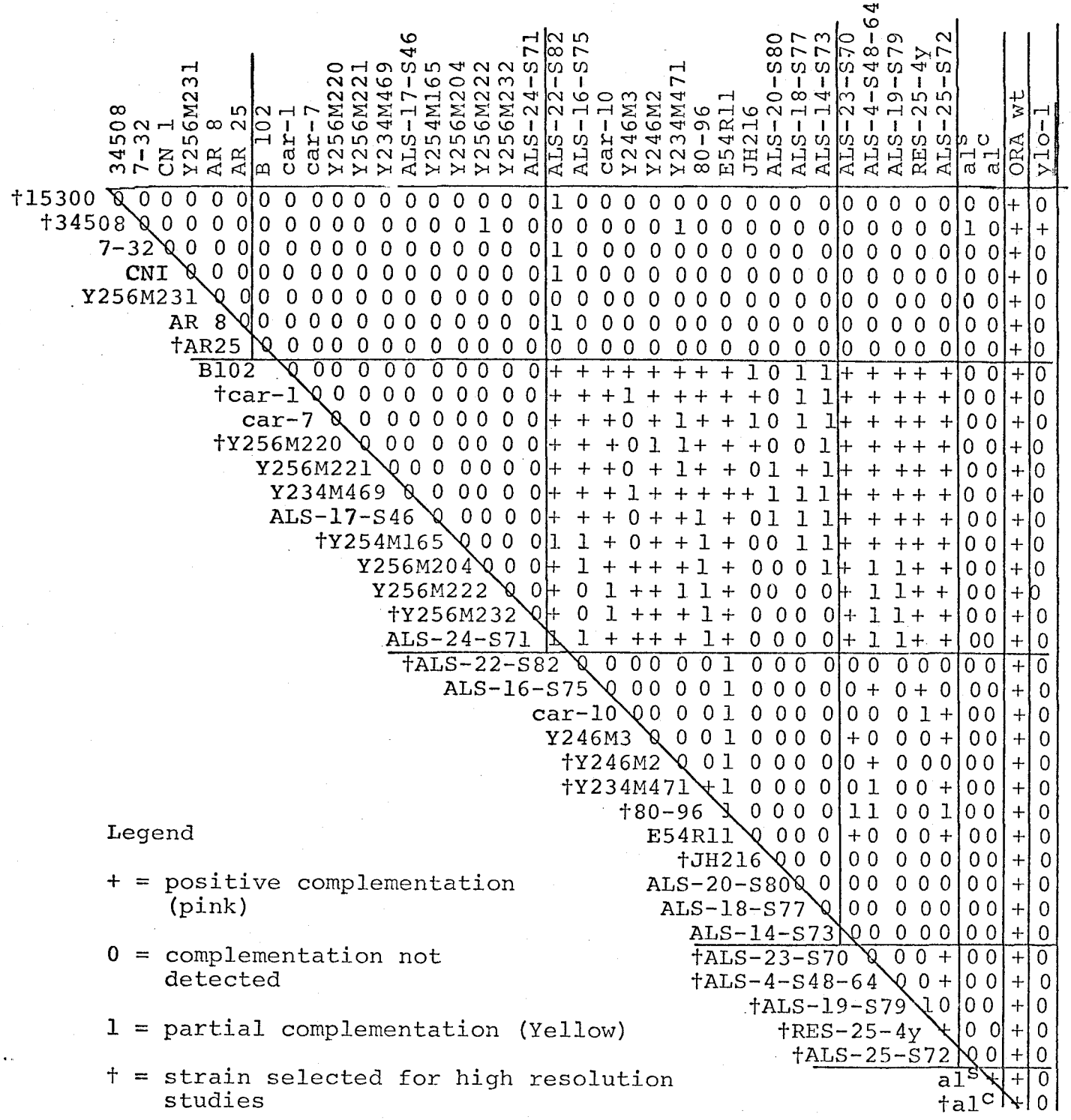
Results from the initial complementation screening led to selection of FM strains for further complementation and recombination studies. Strains were selected if 1) their germination growth and fertility characteristics foretold no technical problems, 2) they were representative of a given carotenoid phenotype, e.g. rose albino - Y254M165, 3) they had been previously studied, e.g. Phase II strains. Strains 46-11-68, AR 25 and G2K30 were selected because they were not classified by the screening process and represented a challenge to the high resolution analysis. Strains designated † in Figure (xxii) were those selected for the synthesis of FM strains.

FM strains served a dual function. Their first use was to force or select for heterokaryon formation in complementation tests between two carotenoidless heteroalleles and the second use was for outside markers in the selective technique for high resolution analysis.

Figure (xxii) reports the results of the dual inoculum FM + FM type heterokaryon complementation tests. The strains at the top of the matrix have arg-6 forcing markers while those on the side have lys-3 forcing markers. The effect of the heterokaryon incompatibility loci (Garnjobst and Wilson, 1956-

Figure (xxii)

Mixed Culture Complementation Results




Legend

\+ = positive complementation (pink)

0 = complementation not detected

1 = partial complementation (Yellow)

† = strain selected for high resolution studies

Failure to complement cannot be ascribed to "0" cultures as there is no proof of heterokaryon formation.

1957) was manifest in the number of poor or no growth cultures These loci were obviously not removed by one or two back-crosses to OR-74-1A.

In Figure (xxiii) the 2 FM configurations were each presented, e.g.

FM1A (15300 *al-2* *arg-6*) + (34508 *aur* *lys-3*) FM10A

FM2a (15300 *al-2* *lys* a) + (Y234M471 *arg-6*) FM19a

in spite of the fact that the same pair of carotenoid heteroalleles were involved. The reason for the seeming duplication is that in some cases one configuration gave results different from the other e.g.:

| | Heterokaryon formed | Carotenoid complementation |
|---|---|---|
| FM1a (15300 *al-2* *arg-6*) + (Y234M471 *lys-3*) FM20a | ✓ | negative |
| FM2a (15300 *al-2* *lys* a) + (Y234M471 *arg-6*) FM19a | ✓ | positive |

This phenomenon illustrates two main features of carotenoid complementation, 1) Only positive results are unambiguous and 2) Even though intergenic (*arg-6* and *lys-3*) complementation may be successful in instituting and perpetuating the heterokaryon, interallelic complementation in the *al* region is not necessarily a corollary. These results may be explained by a model (Fincham and Coddington, 1963; Sundaram and Fincham, 1964) that suggests that the ratio of mutant polypeptides which disturb either the conformation or active

site of a multimeric enzyme is based on the nuclear ratio which in turn is a reflection of the conidial input ratio (Pittenger and Atwood, 1954). It has also been held that a nuclear ratio is selected which permits optimal growth (Woodward, 1959). If this is the case with the FM2a + FM19a and FM1a + FM20a heterokaryons, the same nuclear ratios would not be expected, nor would the same carotenoid complementation characteristics.

Successive conidial isolates from subcultured wt p from high resolution analysis crosses often proved the apparent wt p to be a complementing heterokaryon (see Table X). Presumably these colonies arose as heterokaryons from germling hyphal inosculations, each spore having by chance the correct heterokaryon incompatibility loci combinations and sufficient nuclear interaction to exhibit a wt phenotype. PWT's would be indistinguishable from the foregoing class. PWT's would have fewer heterokaryon incompatibility locus limitations inasmuch as six of the seven linkage groups would be identical in the daughter nuclei rising from the breakdown of the original disomic nucleus. Consequently, all heterokaryon incompatibility loci would be homozygous save those (if any) on linkage Group I.

In other systems whether complementation occurs or not is determined by assigning an arbitrary limit to some parameter such as burst size in phage complementation (Bernstein, Denhardt and Edgar, 1965) or growth rate in

Figure (xxiii)

FM + FM Strain Complementation

| | FM1 15300 | FM15 car-1 | FM17 Y254M165 | FM43 Y256M232 | FM3 RES-25-4y | FM33 ALS-4-S48-64 | FM41 ALS-22-S82 | FM31 ALS-19-S79 | FM35 ALS-23-S70 | FM11 JH216 | FM13 $al^c$ | FM19 Y234M471 | FM39 80-96 | FM9 34508 | FM21 AR25 | FM45 46-11-68 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FM2 15300 | 0 | · | · | · | · | + | · | · | · | · | 0 | 0 | · | + | · | · |
| FM16 car-1 | · | · | · | · | · | · | · | · | · | · | + | · | · | + | 0 | · |
| FM18 Y254M165 | · | · | · | · | · | · | · | · | · | · | · | 0 | · | · | 0 | · |
| FM24 Y256M220 | 0 | 0 | · | · | · | · | · | + | · | · | · | 0 | 0 | + | · | · |
| FM8 RES-25-4y | · | · | · | 0 | · | · | · | · | · | · | · | · | · | · | · | · |
| FM42 ALS-22-S82 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| FM36 ALS-23-S70 | · | · | · | · | · | · | · | · | · | · | · | 0 | · | · | · | 0 |
| FM32 ALS-19-S79 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| FM48 ALS-25-S72 | · | · | · | · | · | · | · | · | · | · | 0 | 0 | · | · | · | · |
| FM12 JH216 | 0 | 0 | · | 0 | · | · | · | · | · | 0 | · | · | · | 0 | · | · |
| FM14 $al^c$ | 0 | 0 | · | · | · | · | · | · | · | 0 | · | · | · | 0 | · | · |
| FM20 Y234M471 | + | · | · | · | · | · | · | · | · | · | · | 0 | · | 0 | · | · |
| FM30 Y246M2 | 0 | · | · | · | · | · | · | · | · | · | 0 | · | · | 0 | · | · |
| FM10 34508 | + | + | · | · | · | · | · | · | · | 0 | · | · | · | 0 | · | · |
| FM28 G2K30 | · | · | · | · | · | · | · | · | · | · | 0 | · | · | · | · | · |

Legend

+ = positive complementation (pink)

0 = no complementation observed in heterokaryon

· = no heterokaryon formed or growth too sparse to classify if carotenoid complementation took place

Neurospora nutritional mutants (Radford, 1966). The limit, where positive complementation starts in some cases is little more than the highest values for one of the mutants. In this study positive complementation was scored only when a characteristic pink colour was phenotypically observable. When two albinos complemented to give a pale yellow pink phenotype, partial complementation was recorded. Visual determination was straightforward except for several of the ylo-b type strains.

The data from the mixed culture screening, the FM X FM complementation tests and the complementing heterokaryons (and PWT's) from the high resolution analysis were all combined in Figure (xxiv).

Attempts to construct a linear, circular or otherwise geometrical complementation map (Fincham, 1966) have met with frustration with several exceptions to continuity. The principle objections emanate from the fact that extensive acceptable negative results are unobtainable with the set of strains used in this system. Mixed cultures cannot be proven heterokaryotic without enlisting other markers or an extensive backcrossing programme. Even if proven heterokaryotic they still required the correct nuclear ratio. FM + FM tests may or may not select for the correct ratio. The wt p hunt gave the greatest number of positive results (complementation from PWT or hyphal anastomoses) but could not be used at all for negative complementation results.

Figure (xxiv)

Matrix of Complementation Data From Mixed Culture PW. T, and FM + FM Strain Heterokaryons

| | Y254M165 | 15300 al-2 | Y256M232 | AR 25 | Y256M220 | car-1 | ALS-19-S79 | ALS-22-S82 | 34508 aur | ALS-25-S72 | ALS-23-S70 | RES-25-4y | ALS-4-S48-64 | Y246M2 | 80-96 | alc | JH216 | Y234M471 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Y254M165 | · | + | + | + | + | 0 | + | + | + | + | + | + | + | + | + | · | · | + |
| 15300 al-2 | 0 | · | · | 0 | + | + | + | + | + | + | + | + | + | 0 | + | + | + | + |
| Y256M232 | · | · | · | · | + | + | + | + | + | + | + | + | + | + | + | + | 0 | + |
| AR 25 | · | · | 0 | · | · | · | · | · | · | · | · | · | · | · | · | + | + | · |
| Y256M220 | · | 0 | + | + | + | + | + | + | + | + | + | + | + | + | + | · | + | + |
| car-1 | · | + | + | + | + | + | + | + | + | + | + | + | + | 1 | · | + | + | + |
| ALS-19-S79 | · | · | · | · | · | · | · | · | · | · | · | · | 1 | · | · | · | · | 1 |
| ALS-22-S82 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| 34508 aur | 0 | · | · | 0 | · | · | · | 0 | · | + | 0 | · | + | 0 | · | + | 0 | + |
| ALS-25-S72 | · | 1 | 1 | 1 | · | · | · | 1 | 0 | · | · | 1 | 0 | · | 1 | 0 | · | + |
| ALS-23-S70 | · | · | · | · | · | · | · | · | · | · | · | · | · | + | 1 | + | · | + |
| RES-25-4y | · | · | · | · | · | · | · | · | · | · | · | · | · | + | + | + | · | + |
| ALS-4-S48-64 | · | · | · | · | · | · | · | · | · | · | · | · | · | + | 1 | 1 | · | + |
| Y246M2 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | + |
| 80-96 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | + |
| alc | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | 0 | · |
| JH216 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| Y234M471 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |

Legend

\+ = positive complementation (pink)

0 = no complementation observed in heterokaryons

1 = partial complementation (yellow)

· = no complementation observed in mixed culture

Based on reliable positive results and the possibly negative results from three independent tests (mixed culture, FM + FM and PWT or heterokaryons from the wt p hunts) a (hypothetical) complementation map was constructed as shown in Figure (xxv).

27. The Biochemistry of the Carotenoid Mutants

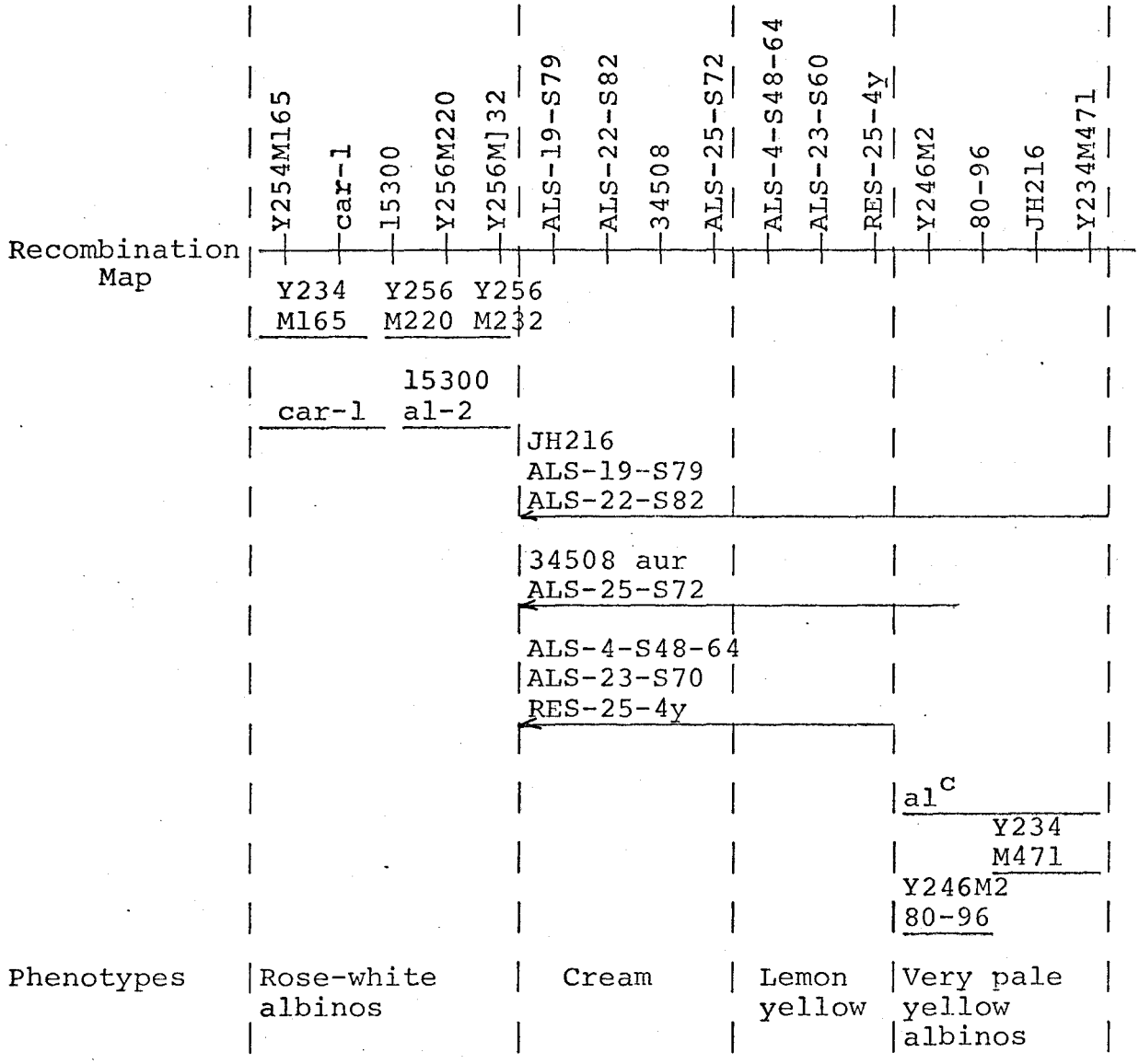
Determination of the weight of the carotenoid fraction in mutant and wt strains was obtained from samples of the acetone homogenates of RES-25-4y and 74-OR8-1a. The weight of the carotenoid fraction was obtained by the procedure described in Section 21. The dry weight of the mycelium was obtained by drying the homogenate sample for two days at 60°C.

The complete spectrum of Neurospora carotenoids was obtained by repeated analysis of the 74-ORA-1a carotenoid fraction. Mutants ylo-1, ylo-b, aur and strain Y234M471 were selected as representative mutants for the four phenotypic groups. The al-2 15300 Group I mutants had previously been analysed (Haxo, 1947). The following carotenoid descriptions relate to pigments obtained from strain 74-OR8-1a.

Phytoene was never identified successfully from any of the fractions collected before phytofluene on the column. An unknown, unidentified compound included in the carotenoid extracts (possibly a steroid) tended to absorb in the 220 mμ to 280 mμ region obscuring the absorption spectrum of phytoene. The existence of this unknown compound had been previously

Figure (xxv)

Postulated Complementation Map*




*Constructed from negative results only from the heterokaryon tests (as opposed to mixed culture) and positive results from any source.

Exceptions to the map { al-2 15300 + Y246M2 ; Y256M232 + JH216 } failed to complement in a FM + FM heterokaryon

TABLE XII

Carotenoid Production*

| | RES-25-4y | 74-OR8-1a |
|---|---|---|
| Run #1 | | |
| dry wt. of mycelium | 33.582 g | 25.113 g |
| wt. of carotenoid fraction | .219 g | .356 g |
| % carotenoid fraction | 0.83 % | 1.29 % |
| Run #2 | | |
| dry wt. of mycelium | 16.119 g | 44.621 g |
| wt. of carotenoid fraction | .091 g | .467 g |
| % carotenoid fraction | 0.56 % | 1.05 % |
| Average % of carotenoid fraction | 0.69 % | 1.2 % |

*wts. shown are for 10 day old cultures.

reported (Bulger, 1966). One study (Karunakaran, Karunakaran and Quackenbush, 1965) using a 2-$C^{14}$-labelled mevalonate phytoene precursor, demonstrated that the "phytoene" pool was only slightly affected by the induction of carotenogenesis. This with other tracer studies had implied that the carotenoids were a minor derivative of phytoene. It is possible that the unknown substance may have contaminated their phytoene extracts giving misleading results.

The intense UV fluorescence of the phytofluene was used to follow the progress of the column front. The band was collected manually in the dark under UV lighting. The absorption spectrum for phytofluene is given in Figure (xxvi). The differences in the absorption between the experimental and expected 332 mμ and 347 mμ maxima were due probably to traces of the unknown substance mentioned in the previous paragraph.

The β-carotene fraction was rechromatogrammed and was difficult to separate from what later proved to be β-zeacarotene. In one instance where the separation was evident an absorption spectrum was obtained (Figure (xxvii)) that correlated well with the literature values. A recent report (Harding, 1968) found no evidence for β-carotene in wt extracts which was possibly due to the fact that he used 3-5 day old cultures versus 10-14 day old cultures used in this study.

β-zeacarotene normally came off the column in the same tube as β-carotene. Persistent manipulations of the β-carotene

concentration (using prepared β-carotene from K & K Laboratories, Plainsview, New York, U.S.A.) in the reference cuvette, allowed for simple differential spectrophotometry which gave the absorption spectrum shown in Figure (xxviii). The differences between experimental and expected ratios of absorption maxima to adjacent minima (peak to trough ratio) was probably due to the presence of isomers of β-carotene (Haxo, Thesis 1947), or other impurities in the β-zeacarotene fraction.

Previous measurements of the ζ-carotene content of Neurospora crassa (Jensen, 1965) indicate the highest individual carotenoid concentration in 5-6 day old culture. ζ-carotene accounted for 28% of the total wt. of the carotenoid fraction (excluding phytoene). Results from 14 day old cultures are consistent with the preceding evidence (approximately 20% of the total wt. of the total carotenoid fraction). Figure (xxix) presents only one absorption spectrum as the experimental and literature spectra were congruent.

γ-carotene and neurosporene were obtained in solution by redissolving the respective bands on the extruded column in hexane (see Section 21). Absorption spectra were not presented, as maximum absorptions of $<0.25$ and the absence of well defined minima rendered results unacceptable.

The bright red band (see Table XIII) appeared to consist of two carotenoids one of which was soluble in a hexane:acetone:water (90:7:3, v/v/v) solution. The remainder

could not be redissolved in hexane:acetone:water solution. The column position and solubility characteristics (Davies, 1965) of the soluble fraction was similar to those of a synthetic lycopene obtained from K & K Laboratories Inc. (Plainsview, New York, U.S.A.). The peak to trough ratio differences between the absorption spectrum of the synthetic and experimental lycopene was probably due to traces of torulene which has similar chromatographic and solubility characteristics (Jensen, 1965). Differences in the absorption maxima are probalby due to the presence of isomers of lycopene. These form by changes of trans to cis configuration double bonds especially at the 15-15' (Weedon, 1965).

The topmost band was completely insoluble and from the column position presumed to be 3,4 dehydrolycopene (formerly spirilloxanthin) traces of neurosporoxanthin and miscellaneous inclusions.

Absorption spectra from the hypophasic fraction revealed a broad based curve with maxima about 8 mμ different from values reported in the literature (Zalokar, 1957) for neurosporoxanthin. This was of little concern as there was only one hypophasic carotenoid present in Neurospora (Haxo, 1947; Zalokar, 1957; Krzeminski, 1959; Jensen, 1965; Harding, 1968).

TABLE XIII

Description of Carotenoids from a Column Chromatogram of N. crassa Strain 74-OR8-1a Extract

| Column | Colour | Carotenoid | Rf in hexane | λ in hexane* (mμ) | | | ε {1%* 1 cm (at major λ max) |
|---|---|---|---|---|---|---|---|
|  | pink | 3,4 dehydrolycopene | 0.05 | 463 | 493 | 527 | 3000 |
| | bright red | torulene | 0.15 | 460 | 484 | 518 | 3200 |
| | | lycopene | 0.15 | 445 | 472 | 504 | 3400 |
| | orange-yellow | neurosporene | 0.20 | 414 | 439 | 466 | 2900 |
| | rose | γ-carotene | 0.45 | 434 | 460 | 491 | 2600 |
| | yellow-green | ζ-carotene | 0.55 | 376 | 398 | 418 | 2000 |
| | yellow | β-zeacarotene | 0.60 | 405 | 425 | 450 | 2500 |
| | orange | β-carotene | 0.62 | 425 | 450 | 478 | 2500 |
| | fluorescent | phytofluene | 0.90 | 332 | 347 | 368 | 1500 |
| | | phytoene | 0.95 | 275 | 286 | 298 | 1200 |
| | red | neurosporoxanthin | - | 450 | 477 | 505 | 1800 |

* from Jensen, 1965.

Spectra are given with the solvent hexane in the reference cuvette. Broken lines indicate absorption spectra from previous authors while solid lines represent experimental spectra obtained in this study.

Figure (xxvi)

Phytofluene UV Absorption Spectrum

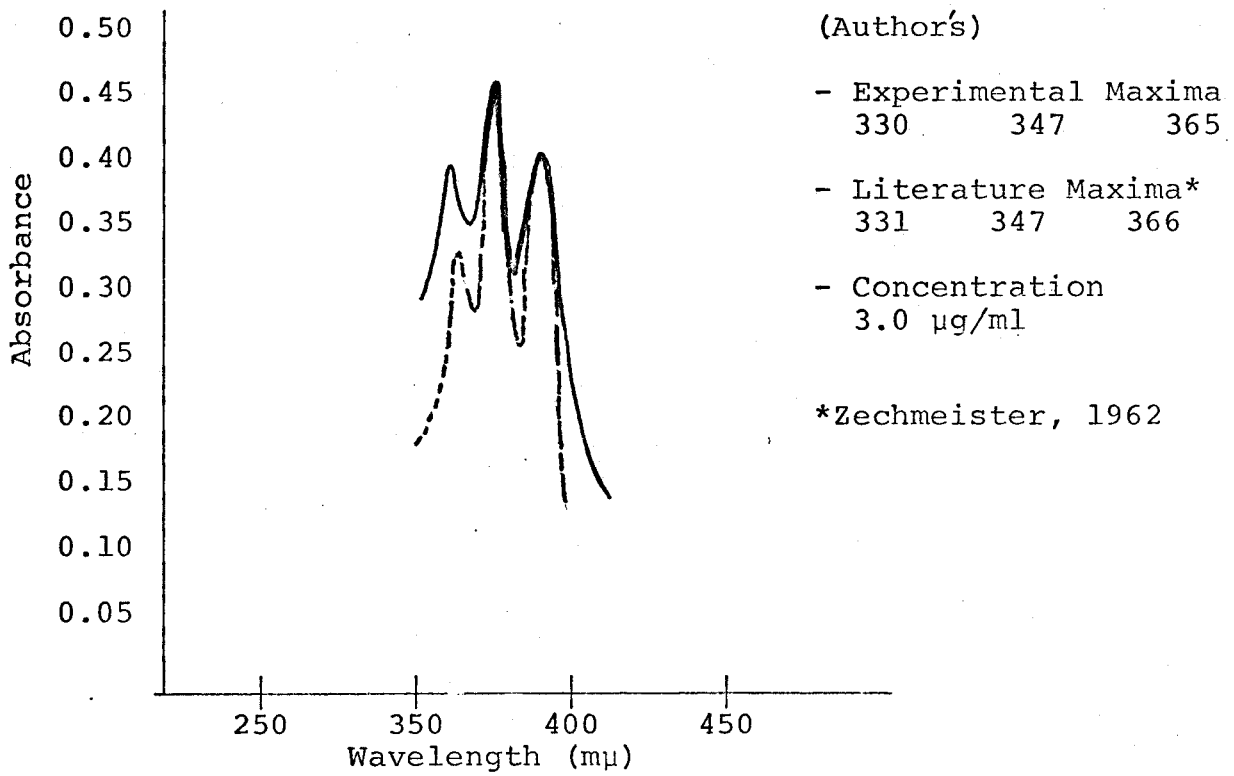
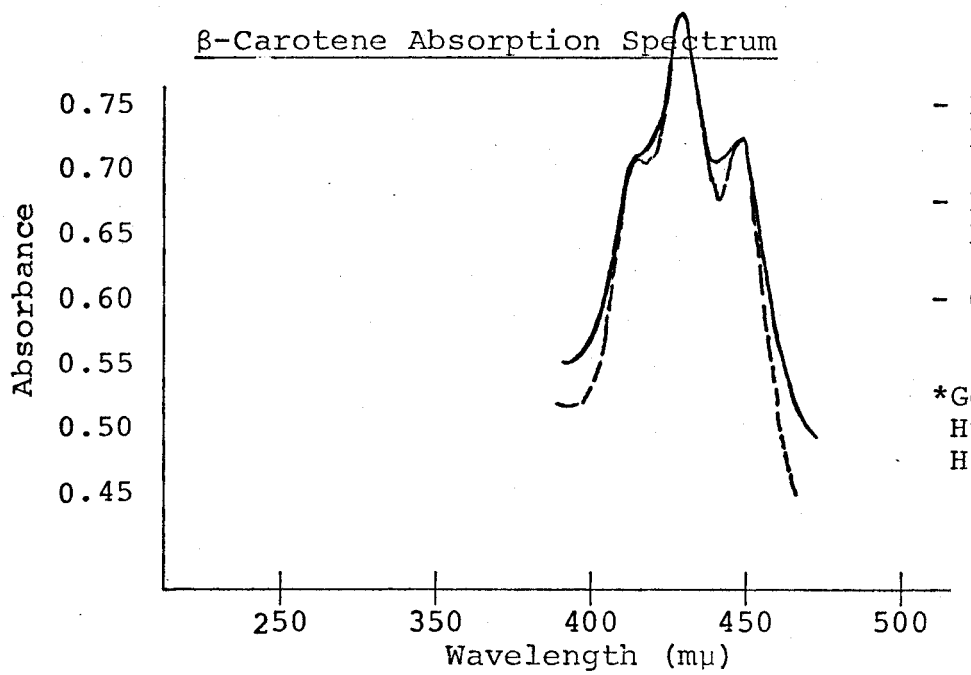


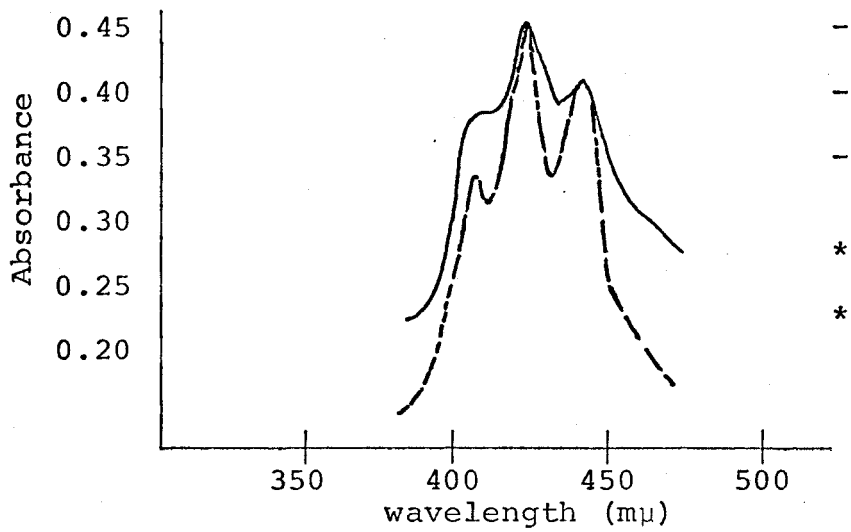
Figure (xxvii)




- Experimental Maxima
  425 453 480
- Literature Maxima*
  425 450 482
- Concentration
  3.1 μg/ml

*Goodwin, 1965
Huang, 1964
Haxo, 1947

Figure (xxviii)



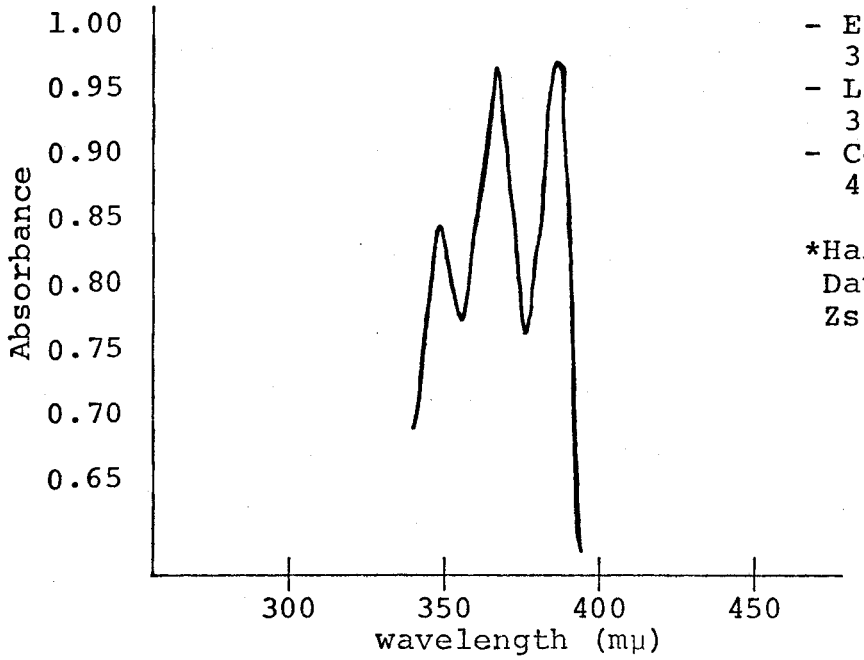

- Experimental Maxima
  408 428 450
- Literature Maxima*
  406 428 454
- Concentration
  1.8 μg/ml

*Isler and Schudel, 196

**reference cuvette contained a β-carotene solution approximately 0.5 μg/ml

Figure (xxix)

ζ-carotene. Absorption Spectrum

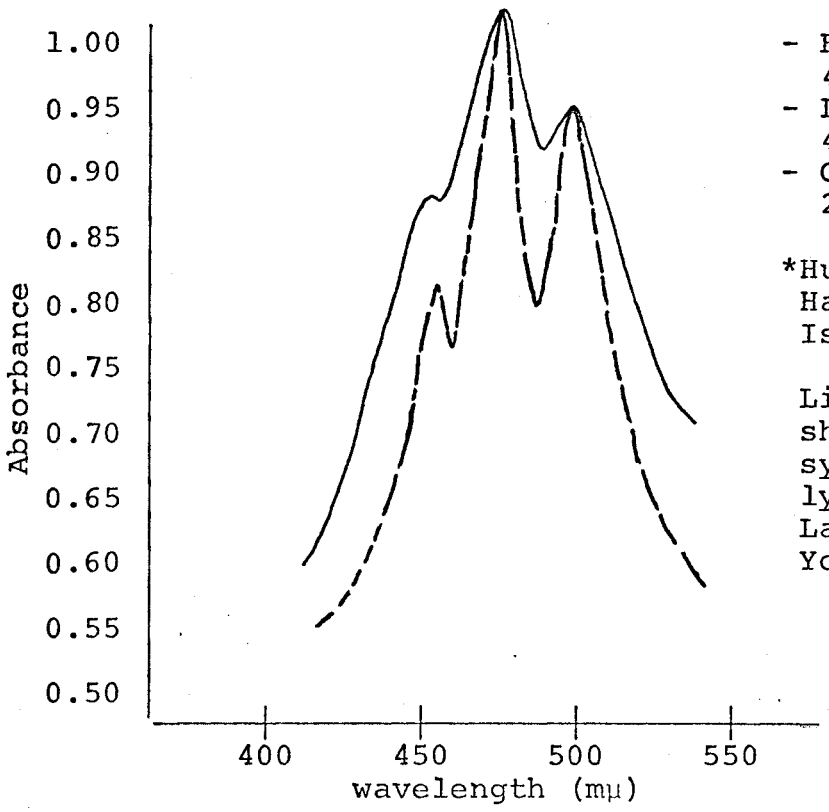



- Experimental Maxima
  375 397 421
- Literature Maxima*
  375 397 421
- Concentration
  4.8 μg/ml

*Haxo, 1947
Davies, 1963
Zscheile, 1945

Figure (xxx)

Lycopene Absorption Spectrum



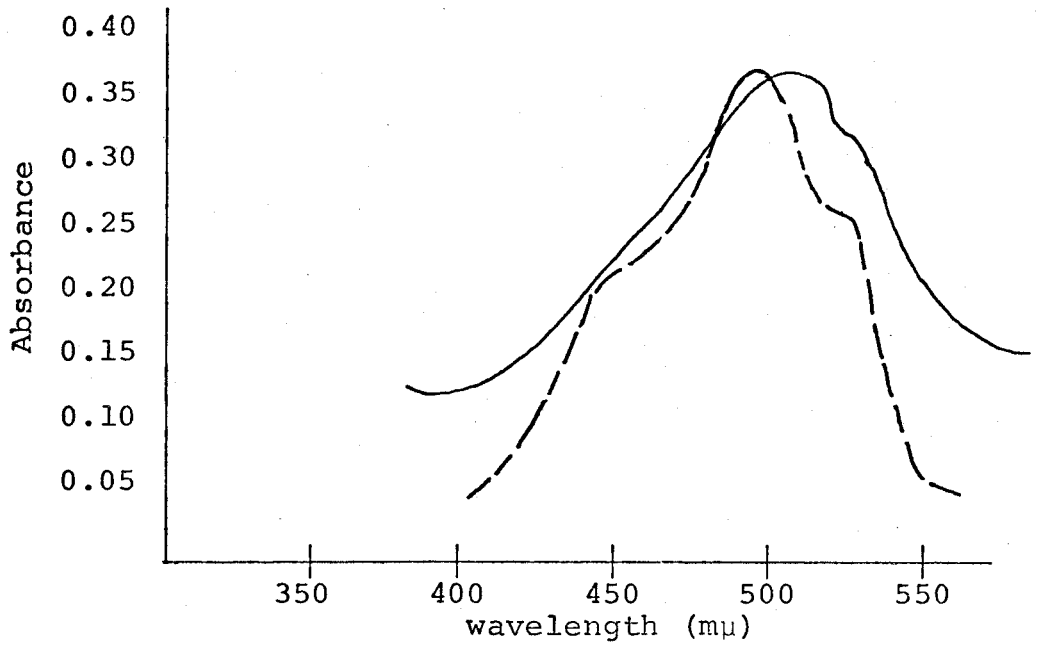

- Experimental Maxima
  449 474 506
- Literature Maxima*
  446 474 506
- Concentration
  2.8 μg/ml

*Huang, 1964
Haxo, 1947
Isler & Schudel, 1963

Literature curve shown in from a synthetic sample of lycopene from K & L Labs, Plainsville, New York, U.S.A.

Figure (xxxi)

Neurosporoxanthin Absorption Spectrum

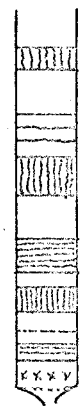
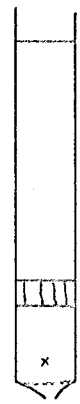
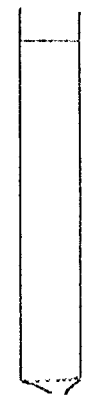



- Experimental Maxima
  490 520
- Literature Maxima*
  482 516
- Concentration
  2.1 μg/ml

*Zalokar, 1957

Figure (xxxii)

Comparison of Carotenoids From Different Mutant Strains

| Strain | 74-OR8-1a | ylo-1 Y30539y | ylo-b RES-25-4y | aur 34508 | al-1 Y234M471 |
|---|---|---|---|---|---|
| Column |  |  |  |  |  |
| Carotenoid Content of mycelium ($\frac{\text{mg carotenoids}}{\text{mg d wt.}}$ %) | 1.2% | 0.83% | 0.69% | 0.07% | 0.0% |
| Carotenoid Distribution* | | | | | |
| phytofluene | 10% | 5 % | 12 % | traces | traces |
| β-carotene | 14% | 18 % | 20 % | 0.0% | 0.0% |
| β-zeacarotene | | traces | traces | 0.0% | 0.0% |
| ζ-carotene | 28% | 32 % | 55 % | 97 % | 0.0% |
| γ-carotene | 4%** | - | - | 0.0% | 0.0% |
| neurosporene | 19%** | 10 %† | - | traces | 0.0% |
| lycopene | 11%** | 21 %† | 13 % | 0.0% | 0.0% |
| torulene | | 12 %† | 0.0% | 0.0% | 0.0% |
| 3,4 dehydrolycopene | 2%** | 2 %† | - | 0.0% | 0.0% |
| neurosporoxanthin (not shown) | 10% | <0.3% | <0.2% | 0.0% | 0.0% |
| unknown | 2% | - | - | 2 % | 0.0% |

*Individual carotenoid amounts given as a percent of the total molar concentrations for all carotenoids combined.
**from calculations based on results from Jensen(1965)
-means that an absorption spectra for the individual carotenoid was not detected.
traces - refers to quantities visible on the column but beyond the resolution of the experimental procedures.
†from calculations based on results from Harding (1968).

The results shown in Figure (xxxii) confirm the position designated by Haxo (1947) for the ylo-1 block i.e. prior to the synthesis of neurosporoxanthin. High concentrations of torulene and lycopene (2 and 3 times wt concentrations) are in agreement with the idea that because of the block some of the precursors are rechannelled at a branch point earlier in the carotenogenic pathway (see Figure (xxxiii)).

The block caused by the ylo-b mutation is placed prior to the synthesis of torulene. The tentative identification of torulene is based on three criteria:

1) Previous reports (Jensen, 1965; Harding, 1968) have found torulene from Neurospora carotenoid extracts at column positions similar to those shown in Table XIII.

2) Adsorption afinity on alumina (Davies, 1965) indicate that torulene is more tightly bound to the column alumina than lycopene.

3) $R_f$ values (0.20 lycopene, 0.15 torulene in hexane on deactivated alumina, Goodwin, 1965) allow for overlapping bands on the column. Because neurosporoxanthin is present only in trace quantities it follows that neurosporoxanthin is possibly synthesized from torulene.

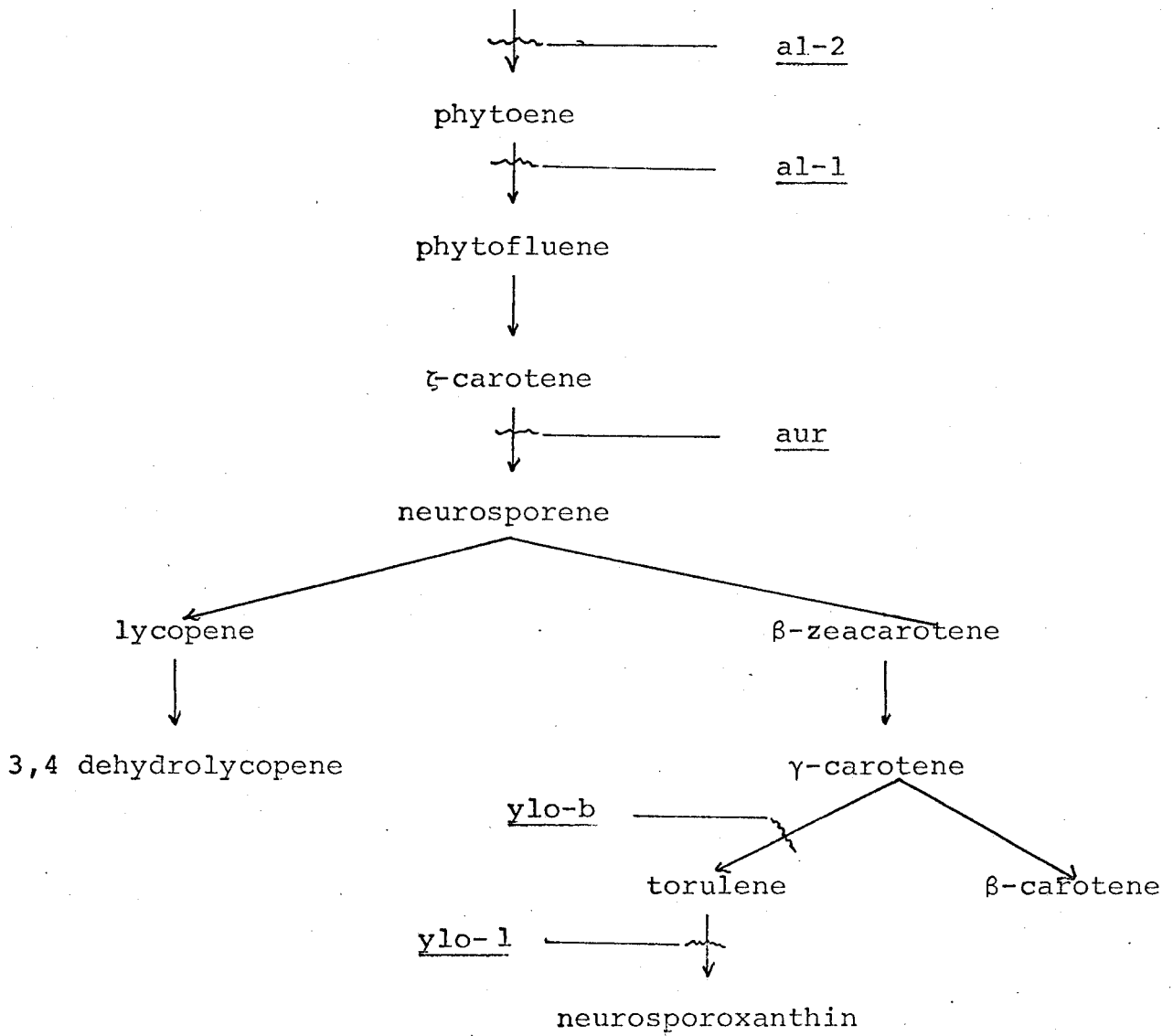
Aurescent strains (34508) produced very low carotenoid yields (0.07%). Phytofluene and ζ-carotene were produced in detectable quantities. Neurosporene had been found in aur extracts but only in quantities attributable to gene "leakiness" (Harding, personal communication) therefore the

block effected by aurescent was placed before neurosporene.

The al-1 type mutants represented by Y234M471 showed no carotenoids except traces of phytofluene. Haxo (1947) placed the al-1 type mutant block before phytofluene and the al-2 type mutants before phytoene. Haxo's results for al-2 (15300) and al-1 (Y234M471) type mutants are used in Figure (xxxiii)

Figure (xxxiii)

The Proposed Carotenogenic Scheme Blocks



28. Analysis of Strain P1193, a ylo-1 Allele

Strain P1193 expressed a phenotype identical to ylo-1 which is located on linkage group VI L (Barratt and Ogata, 1964). One hundred random spores from a ylo-1 a X P1193 A cross produced no wt s indicating allelism. Four strains were synthesized for high resolution studies as shown in Figure (xxxiv). A selective technique similar to that used for the al region was employed. The approximate number of viable spores is calculated from the published map relations as follows:

ylo-1 to ad-8 interval 25 cou (Ishikawa, 1961)
ylo-1 to tryp-2 interval 20 cou (Threlkeld, 1961)
ylo-1 to cys-1 interval 10 cou (Shen, 1950)
ylo-1 to lys-5 interval 15 cou (Stadler, 1956)

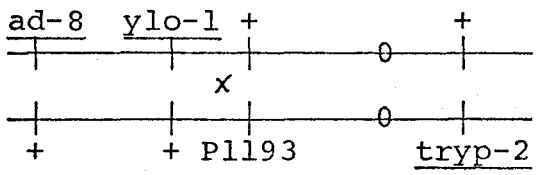
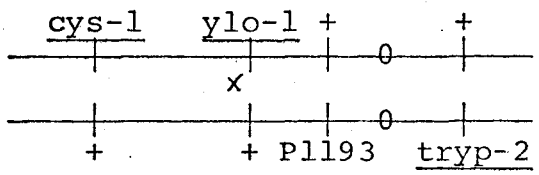
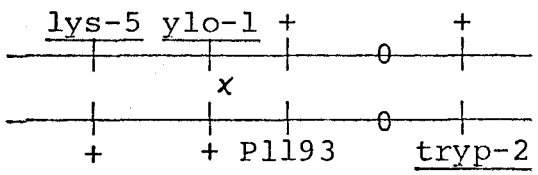
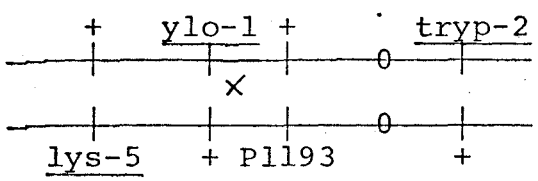
Because colour discrimination was difficult the plating density was reduced from the 16 colonies per plate used in the al region study to 10.

The results from Figure (xxxiv) prove that the two alleles are very close together (< 0.01 cou). One wt p from 22000 viable spores although not significant permits us to tentatively locate the allele proximal to ylo-1 (Y30539y).

Carotenoid extracts of P1193 gave evidence for only trace quantities of neurosporoxanthin similar to ylo-1.

Figure (xxxiv)

Results of High Resolution Crosses for P1193

| | wt p | total prototroph | approximate number of viable spores |
|---|---|---|---|
|  | 0 | 3242 | 14000 |
|  | 0 | 455 | 3000 |
|  | 1 | 515 | 3000 |
|  | 0 | 36 | 240 |

# DISCUSSION

## 29. Mapping

From the recombination results summarized in Figures (xx) and (xxi) it would appear that the al region on the left arm of linkage Group I in Neurospora crassa is one complex locus $\leq$ 1.5 map units in length (the map distance between the most distant markers) containing two major subunits. The first subunit consists of a cluster of closely linked mutant sites $\sim$ 0.2 map units long and expressing an albino phenotype with a faint rose cast. The second subunit is separated from the first by a map interval of $\sim$ 0.5 map units. The phenotypes "cream" albino, lemon yellow and albino with a very faint yellow cast were expressed in a proximal to distal order in the second subunit which occupied $\sim$ 0.5 map units. For reference purposes the phenotypic groups were designated al-2 (for the first subunit) and aur ylo-b al-1 in the second subunit in proximal to distal order. The interpretation of the results from the recombination data was relatively straight forward but comments on resolution ordering versus map distance for site assignation, negative interference, PWT effect and special strain effects bear special consideration.

In considering the degree of resolution available it

may be assumed that if 3000 viable spores are plated per cross then one wt p in 3000 spores or 0.06 map units would be the system's basic resolving power. In several instances in the recombination maps Figures (xix) and (xx) the prototroph frequencies were given as 0.0/0.0 . This would mean that the map distance between the two alleles was less than 0.06 map units (based on 3000 viable spores). Greater resolution was attempted with several crosses, e.g. 15300 lys-3 X car-1 arg-6, ALS-19-S79 arg-6 X 34508 lys-3, AR25 arg-6 X Y254M165 by repeating the plating runs. Such reruns often produced results inconsistent with the established map relationships inasmuch as the order given by the selective technique was not that indicated from the map distance approximations. This condition may have been a reflection of the actual mechanism of recombination (e.g. conversion) between closely linked alleles. Once an allele was located in a cluster, further work (repeated platings) yielded correspondingly less information so an arbitrary cut off at 6000 spores was set. This corresponded to a maximum resolution of 0.03 map units.

One factor that may have affected the validity of map distance approximations (page 67) was the spore viability. If selection existed for viability of the prototroph, then stating the prototroph frequency as a parameter of the viable spore frequency is not valid. The following results indicate slightly lower experimental scores than those expected on the

basis of the counts made for the statistical analysis (Section 25).

| | Spores Plated | Viable Spores | Germination % | Total Prototrophs | Colonies per Plate |
|---|---|---|---|---|---|
| Expected (see Section 25) | 3000-4000 | 3080 | 88 ± 6.5% | 400 | 16 |
| Observed (mean from Table X) | 3500 | 2780.9 | 79.4 ± 6.5% | 317 | 12 |

The aforementioned "observed" figures are averages that exclude reversion strains yet include strains such as Y254M2, 46-11-68, AR 25 and RES-25-4y which gave consistently low spore yields (<3000 spores) lowering the average prototroph yield. Even so the difference does not indicate a significant difference from the expected frequency.

As previously mentioned on page 79 the orders indicated by the selective technique superceded those indicated by the map distance approximations even though alternatives other than a classical single cross over were possible to explain wt p's. The most plausible alternative was the presence of high negative interference (Pritchard, 1955; Elliot, 1960; Chase and Doermann, 1958; Sherman and Roman, 1963) which would appear as an apparent excess of the triple crossover wt p. The expected frequency of single crossover wt p to triple crossover wt p was 400:1 (see Sections 2 and 25, Figure (xvi)). The observed frequency was 31 :1 which differed significantly

from the expected ($\chi^2$ 6.1 $p < .05$.) This frequency was obtained from the actual number of wt p's and contradictory wt p's as shown in Figure (xx) excluding $\underline{al}^c$ and RES-25-4y which will be discussed later in this section. High negative interference has been variously explained through "regions of effective pairing" with copy choice DNA replication (Pritchard, 1955; Freese, 1957a,b) or may be accommodated in the hybrid DNA polaron model (Whitehouse, 1965) through conversion events. It is reported to increase prototroph frequencies from 50 to 100 times the frequency expected on classical cross over theory with no allowance for interference (Case and Giles, 1958a,b).

It should also be mentioned that reports also exist (deSerres, 1956) demonstrating the absence of high negative interference between closely linked sites viz ad-3A - ad-3B.

The diminution of the map distance (from that previously published by Hungate, 1945; Huang, 1964) separating Group I and Group II alleles (approximately 2-4 map units to $\underline{\sim}$ 0.5 units was accounted for by suggesting that previous authors had neglected to perform successive conidial isolates on their apparent wt p's to preclude the inclusion of data from complementing heterokaryons either from PWT's of spore hyphal anastomoses.

The possibility of PWT's presented an interesting situation inasmuch as heterozygosity at the mating type locus results in bizarre growth ending in vegetative atrophy

and media discolouration (Newmeyer and Taylor, 1967). Two exceptional cases have been reported (Lindegren, 1934; Gros, 1952) but the strains were self sterile. PWT's involving linkage Group I, isolated by Pittenger (1954) were of the same mating type. Clearly heterozygous mating type PWT's were selected against or a single cross over may have occurred between the centromere and the MT locus prior to a second division non-disjunction.

Special strain effects are generally due to genic or chromosomal factors. Determining the specific nature of the following effects is not within the scope of this thesis so the report is confined to a description of the effect and the most probable explanation of the effect based on the available evidence.

1) RES-25-4y lys-3 a produced wt p's from crosses with distal al's in FM arg-6 strains and a wt p from a homoallelic cross. This is most likely due to a reversion of the ylo-b marker. The reversion nuclei if present at a sufficiently low frequency would have their wt phenotype masked by the ylo-b phenotype.

2) $al^{c}$ arg-6 produced wt p's from crosses with proximal al's in FM lys-3 strains and an inconsistent pattern of recombination was established wherein two sites were possible (proximal or distal to aur). Some crosses (see Page 71) produced morphologically atypical progeny and hyaline spores which often indicates some chromosomal anomally. Some chromosomal aberration

may exist between $al^c$ and crisp. The mutant site was mapped by reference to $al^c$ lys-3 results.

3) ALS-22-S82 produced no recombinants or positive complementation with any distal carotenoid marker. The effect is presumably due to a deletion or inversion.

4) 46-11-68 produced few perithecia and very few spores. Three crosses (Table X) were exceptional inasmuch as they yielded normal ascospore harvests. There is a possibility that crossing, like heterokaryon formation requires a certain genetic combination (including the mating type locus) and that a gene complement permitting crosses was realized in the three exceptional crosses only.

5) Strains car-1 and AR 25 produced consistently low recombination values with distal markers. This may be due to the incorrect assignment of the mutant sites (should be distal to Y256M220) as a result of triple cross over effect on ordering or possibly that $rec^+$ genes (Jessop, Catcheside, 1965; Griffiths, 1968) or other background effects have predisposed the strains not to undergo crossing over.

6) Strains $al^c$, JH216 and E54R11 expressed a crisp-like morphology when crossed out of their original backgrounds. No immediate explanation seems reasonable other than it is possible that the carotenoid locus products or loci closely linked to the carotenoid loci may be involved in changes in primary glucan-glucosamine metabolism responsible for this type of mutant morphology.

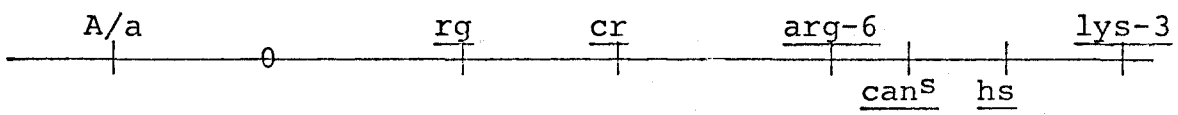
30. Complementation Discussion

To answer the question "Is the al region two discrete loci associated with the same function, two discrete loci associated with different functions in the same pathway or one locus containing a cluster of cistrons with functions associated with the same pathway?" requires extensive information from recombination, biochemical analysis and complementation studies and it would appear that the complementation results suffer the greatest interpretative ambiguity. The problems of carotenoid complementation are described in Section 19 and summarized by P. C. Huang (1964), and in general terms reflect a condition where only positive complementation was completely unambiguous. A complementation map so formed would be a meaningless one dimensional string of complons.

If one accepts the evidence from mixed cultures and heterokaryons for non-complementation a classical (Fincham, 1965) linear map with few incidences (15300 al-2 + Y246M2 and Y256M232 + JH216) of discontinuous complons is possible (see Figure (xxv)). Several of these exceptions (discontinuous complons) may be avoided if a circular map is constructed (as previously noted by Kapuler and Bernstein, 1962) or if the map is made up of two adjoining circles. However, such manipulations with the complon geometry fail to add significantly to information concerning the mode of action of a locus (Crick and Orgel, 1962; Gillie, 1968; Kapuler and Bernstein, 1963)

Although preliminary studies on the data from Figure (xx) and (xxiv) indicated a possible operon-like organization for the al region similar to the hist-3 complex (Ahmed, Case and Giles, 1964), a more detailed study revealed that a complex locus of two independent cistrons was more likely. One of the cistrons (Group II) would be polyfunctional performing successive dehydrogenations on the same substrate molecules. The failure, due possibly to negative complementation (Fincham, 1965), of some Group I plus Group II strain heterokaryons to exhibit a wt phenotype (Y254M165 + $al^{c}$, 15300 + Y246M2 and Y256M232 + JH216) may be interpreted as some form of an enzyme aggregate not unlike fatty acid synthetase (Lynen and Tada, 1966; Grant, 1963).

In retrospect the problems of heterokaryon incompatibility and the synthesis of suitable FM strains, might have been overcome if mutants had been obtained by UV irradiation of a "ragged crisp arg-6 lys-3" strain in an OR background.




The growth is colonial with good conidiation so selection of the "forward" mutation (to $al^{-}$) would be facilitated. The al' arg-6 and al' lys-3 strains could easily be synthesized with backcrosses to a canavine sensitive and a homoserineless strain then plating the progeny on media containing arginine plus canavine (for the al' arg-6 strain) and lysine (for the al' lys-3 strain).

31. Biochemical Implications

Conclusive biochemical evidence exists from the analysis of Y3059y and P1193 to classify them as neurosporoxanthinless mutant strains inasmuch as neurosporoxanthin is the only carotenoid not synthesized by these strains assuming that there is only one hypophasic carotenoid (see page 93). Recombination and complementation results confirm this evidence, describing them as two non-complementing, closely linked (<0.01 units) sites approximately six units from the centromere on the left arm of linkage Group VI.

The mutant phenotypes represented by RES-25-4y can be tentatively ascribed to an inability to synthesize torulene and neurosporoxanthin. Mutants expressing this phenotype, map in the al region of the right arm of linkage Group I as do the following mutants.

It is not clear from the biochemical evidence whether aur is a very "leaky" mutant with a primary effect on phytoene dehydrogenation or if carotenogenesis in toto is depressed and the primary effect is on ζ-carotene dehydrogenation.

Mutant strains expressing an al-1 type phenotype produce phytoene in quantities characteristic of wt strains (Huang, 1963; Haxo, 1947; Harding, 1968) but little or no phytofluene. This would indicate that the primary effect of the al-1 mutants is on phytoene dehydrogenation.

Ambiguities in ascribing mutant effects were present in the assignment of al-2 type strains. Early reports (Haxo,

1947) deny the existence of quantities of phytoene other than that attributable to gene leakiness. Later reports (Huang, 1963; Harding, 1968) report its presence in al-2 type strains in quantities slightly less than that in wt strains. This inconsistency may be due to the experimental error in estimating the phytoene pool size because of the unknown substance that absorbs in the same region as phytoene (see Section 27) although Huang (1964) did report a similar compound in his albino extracts.

Harding (1968) noted that carotenogenesis was light induced, so he postulated that the al-2, al-1 phenocopies were due to one group being concerned with the light response that in turn induced the second group to synthesize the phytoene dehydrogenase. In this manner complementing groups were possible but non-complementing Group I - Group II pairs were puzzling except if viewed as aninteraction either at the gene level or possibly the interaction of polypeptides in a heteromultimer.

These preliminary investigations into the range of carotenoids produced by mutant strains (especially those associated with early blocks) require further sophistication in chromatographic techniques for unequivocal identification of the carotenoids before a conclusive correlation can be made with the complementation and recombination maps.

## APPENDIX A

### Mite Control for Neurospora Labs

As far back as 1939 (Lindegren, 1939, Bot. Gaz. 100: 592) mites have been known to infest and contaminate fungal cultures. Mites are also known to infest Drosophila cultures. Most of the mites concerned are of the genus Tarsonemus (Fig. 1) and a few are Tetranychus or Tyroglyphus. Being phytophagous they eat Neurospora culture in one tube, proliferate there, and then migrate to the next. Because they are only 130 μ long they can enter cotton plugged test tubes and flasks or covered petri dishes. They carry conidia of contaminants or other Neurospora stocks rendering all of the laboratory stocks suspect with respect to their genetic integrity.

Lindane of gammexane (1,2,3,4,5,6-hexachloro cyclohexane) has been used but is only moderately effective and being toxic as well as carcinogenic, is quite dangerous. Sodium or ammonium hydroxide vapour will clean out an incubator but apparently does not affect established mite colonies. Dipping the plugs in glycerine is effective but tedious and messy.

The proposed method of an absolute control programme consiste of two parts: (1) killing all adults, nymphal instars and eggs by freezing, (2) chemical prophylaxis.

Mites will supercool to about -10°C whereupon a crystal lattice forms throughout the body, followed by a decrease in translucence or increase in opacity. The kill remains low at temperatures above -15°C, so care should be taken to treat stocks at -17/8°C (0°F). Presumably the effect is chemical rather than physical (Salt, 1961, Ann. Rev. of Ent. 6: 64) so the rate of cooling is unimportant although for our experiments the rate is described in (Fig. 2). To achieve 100% kill and prevent selection for freeze-resistant or cold-hardy mites, 72 hrs should be the minimum freezing time (Fig. 3). Conidia have almost 100% survival (even at -180°C). Ascospore survival following treatment at this temperature (-17.8°C) is poor. However, crosses that are mite infested are generally rejected so ascospore survival is less important than conidial survival.

Prophylactic measures consist entirely of treatment with Kelthane AP (1,1-bis (chlorophenyl) 2,2,2-trichoro-ethanol), which can be obtained from any Rohm and Haas dealership or from their head office in Philadelphia. It has the desirable properties of: low $LD_{50}$ for mites, high $LD_{50}$ for man, a specific acaracide (has little effect on most insects or even related mite genera) and can be obtained in a wettable powder that is not irritating to the skin. Toxilogical investigations on Kelthane were carried out at the Medical College of Virginia (Smith et al. 1959, Tox. and Appl. Pharm. 1: 119). All work to date has revealed

encouragingly good results (1/4 the oral toxicity of DDT to mammals). It is reported that, although residual effects of Kelthane on mites is fair, a bi- or tri-annual treatment is recommended. Culture and cross racks, incubator shelves and walls, and window perimeters should all be wiped down with Kelthane AP. This acaracide affects only the adults so it should be carried on in conjunction with the freezing programme which kills the eggs. Tarsonemus is particularly sensitive to dryness, so dehymidifiers are also preventitives.

Absolute control of infested cultures can be obtained by freezing at -18°C for 24 hrs. and Kelthane treatment of all equipment except tubes, plugs and media.

Figure 1

Male Mite

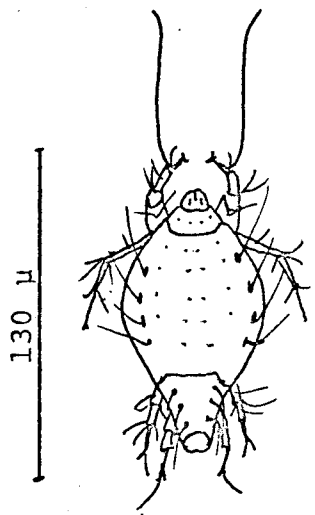


Figure 2

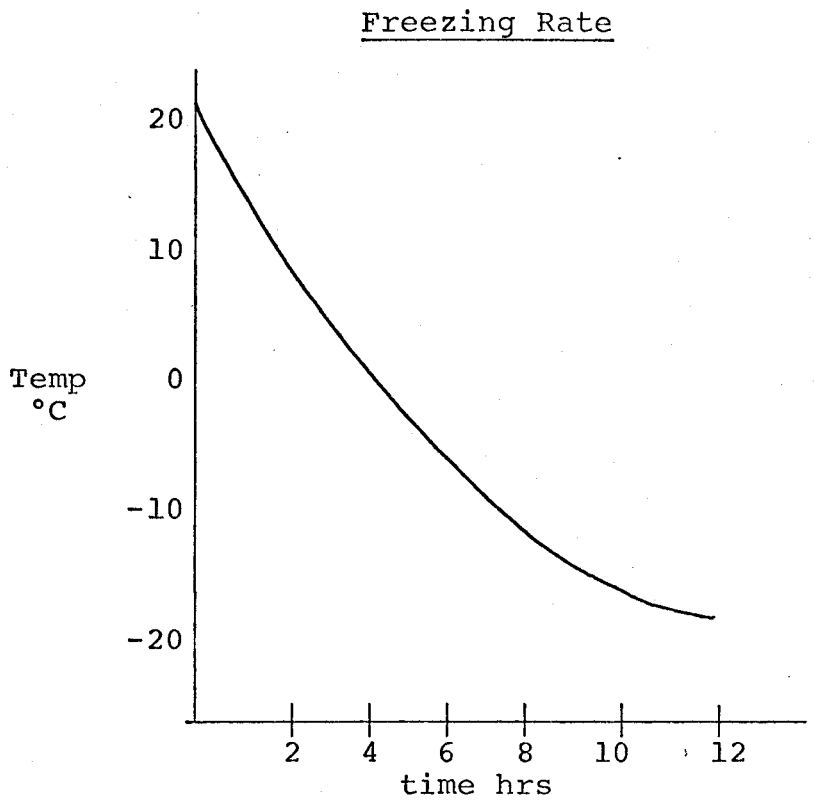
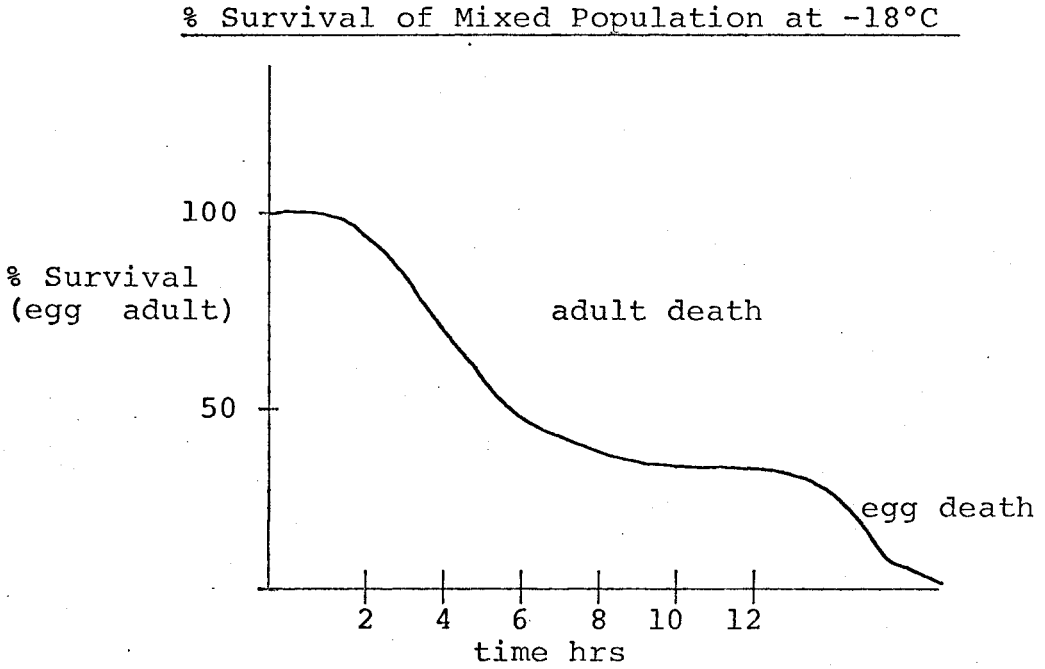



Figure 3



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