SOME GENETIC INTERRELATIONS OF METHIONINE MUTANTS OF NEUROSPORA CRASSA

A Thesis

submitted by

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SYNOPSIS

Methionine mutants of Neurospora crassa were isolated by a filtration concentration technique following ultra-violet irradiation. These and other mutants were classified by the physiological tests of precursor utilisation and heterocaryon complementation.

Precursor utilisation data are in agreement with the present outline of methionine synthesis from cysteine but provide little information on the pathway of sulphate reduction and incorporation of sulphur within a carbon skeleton to yield cysteine.

Seven cysteine and eight methionine loci are represented on the basis of heterocaryon complementation tests. Four of the cysteine loci and either one or two of the methionine loci, associated with the methylation of homocysteine, have not been reported previously.

Two of the eight methionine loci had already been located but five of the remainder were associated with definite chromosome regions. No evidence for the tight clustering of either these or the cysteine loci

was obtained.

Inter-allelic complementation has been observed at two loci and the fine structure of one of these loci (me-2) has been studied in some detail by analysis of prototroph formation in inter-allelic crosses. The use of "outside markers" has facilitated the demonstration of intra-genic recombination and "conversion". Furthermore, these studies provide evidence for a linear arrangement of mutable sites within the locus with additive prototroph frequencies to yield a site map which can be correlated with a complementation map. The results also show that some alleles are more prone to "conversion" than others.

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General Introduction

The ascomycetous genus, Neurospora, was discovered by Sheer and Dodge (1927) who described three species including Neurospora crassa. This species, in common with the other members of its genus and with many other Ascomycetes, shows a number of characteristics which are especially advantageous in genetics studies.

For the major part of its life history Neurospora crassa is haploid so that the complications of dominant and recessive alleles and of allelic interaction do not arise although they can be studied in heterocaryons. This species is also heterothallic enabling crosses between different strains to be made without risk of self-fertilisation.

After fertilisation nuclear fusion occurs in the young ascus and is followed by a meiosis resulting in four haploid nuclei which then undergo mitosis and the eight daughter nuclei are each included in a separate ascospore. Cytological examination of ascus formation (Wilcox, 1928; McClintock, 1945; Singleton, 1953)

revealed that all the products of a single meiotic division are preserved in a very orderly manner within the ascus and bear a precise relation to the planes of the nuclear divisions. The pairs of adjacent spores are found to be genetically identical being sister spores of the mitotic divisions (Wilcox, 1928; Lindegren, 1933) and as the meiotic spindles do not overlap, determination of the segregation division for a particular pair of factors is possible by isolation of the ascospores in order. In contrast to unordered tetrads the centromere may now be used as a point of reference because the centromeres of pairs of homologous chromosomes separate undivided at the first anaphase i.e. first division segregatiom. The frequency of second division segregation is characteristic for any particular gene and is a function of its distance from the centromere.

These properties combine to make Neurospora crassa an excellent organism for genetic study and this was first realised and exploited by Dodge (1929, 1930, 1931). The ease with which pure cultures can be maintained on simple artificial media and the short sexual generation

times of the organism have further aided the development of Neurospora genetics.

The existence of a gene is usually inferred when the loss or alteration of the normal, or wild type, form causes an observable phenotypic change and this difference behaves in a Mendelian fashion. Changes which are quantitatively too small for detection or alternatively so drastic as to prove lethal are overlooked. The observable mutations, representing only a fraction of the actual ones, involve alteration or partial inactivation of essential genes or complete loss or inactivation of non-essential genes. Mutant genes are the "markers" necessary for any genetic experiments but those available for study are limited by the techniques of detection. In Neurospora, and many other microorganisms, where essential metabolites can be supplied in an artificial medium, it is now possible to use many biochemical mutants as "markers" and to study the genetic control of essential metabolic syntheses.

Spontaneous mutations are continually occurring with a very low frequency but by use of X-rays,

ultraviolet light, and chemical mutagens it is possible to increase the mutation rate and obtain large numbers of mutants requiring a variety of amino acids, purines, pyrimidines and vitamins (Beadle and Tatum, 1941, 1945). Mutational change can be associated with a defective step in a synthesis or with a morphological change and has been recognised as the loss of an enzyme (Mitchell and Lein, 1948; Fincham, 1951; Yanofsky, 1952) or as a change in the physical properties of an enzyme as in the case of the thermolabile tyrosinase (Horowitz and Fling, 1953).

The gene, recognised by virtue of its mutated form, can, therefore, be associated with a particular function and referred to a locus in a chromosome linkage map. On the assumption that a mutant locus can be correlated with a defective step in a biosynthesis, a mutant can utilise only those precursors occurring after the resultant blockage. Srb and Horowitz (1944) first demonstrated an ordered sequence of genetic blocks by the study of fifteen arginine mutants, of independent origin, representing seven loci. Mutants at four of the seven loci grew equally well on ornithine, citrulline

and arginine; two grew only on citrulline and arginine and one only on arginine. From these facts they deduced that the order of synthesis was → ornithine → citrulline → arginine.

Wild type Neurospora crassa can utilise inorganic sulphate as its sole sulphur source, other than biotin, which is a vitamin required in minute amount. Many mutant strains are unable to utilise inorganic sulphate, for the synthesis of methionine, but will grow when sulphur is supplied in the amino acid methionine. These mutants, termed methionine mutants, may have a specific requirement for methionine, but are more often able to utilise various precursors of methionine. Although knowledge of the biosynthetic pathway of methionine is still incomplete it is generally agreed that cysteine, cystathionine and homocysteine are precursors (Horowitz, 1947). The biosynthesis of cysteine from inorganic sulphate is more obscure but most data are consistent with an inorganic pathway of sulphate reduction through sulphite and thiosulphate before the reduced sulphur atom is combined in a carbon chain to form cysteine (Horowitz, 1955; Clowes, 1958).

In the discussion following a paper by Demerec (1955), Horowitz pointed out that in Neurospora crassa there was evidence for some of the genes controlling cysteine and methionine synthesis occurring in clusters in linkage groups IV and VI respectively. Most evidence, in organisms other than bacteria, does not support an assembly-line arrangement of cistrons controlling the steps in a sequential process although Pontecorvo (1959) emphasises that there is some evidence for non-randomness of gene distribution. Close linkages between pairs of genes obviously related in action as well as between pairs of genes not so obviously related are encountered more often than would be expected on the basis of a random distribution of genes amongst the chromosomes. However no situation similar to that in Salmonella, where all the tryptophan (Demerec and Hartman, 1956) and histidine (Hartman, 1956) factors respectively are closely linked in the order of action, has yet been established. It was decided, therefore, to examine the possibility that the genes controlling methionine synthesis in Neurospora crassa provide examples of functionally related

groups of genes closely linked in order on the chromosome.

The major part of this thesis is concerned with the investigation of this possibility. By the isolation and classification of a large number of methionine mutants it was hoped to discover any previously unknown loci, to map the loci not previously located, and to study the fine structure of one of these loci.

Materials and Methods

Mutants

The stocks of methionine mutants comprise twelve of Beadle and Tatum's (1945) mutants at nine different loci and 172 new ones. The previously reported mutants are 35809 at locus me-6; 38706 and 35599 (me-1); H98 and 48004 (me-2); 36104 (me-3); 9666 and 86304 (me-5); 39816 (me-4); 84605 (cys-1); 38401 (cys-2) and 39103 (no locus number given) (Emerson, 1950; Barratt et al., 1954; Stadler, 1956).

Of the new mutants 163 were isolated in this laboratory following ultraviolet irradiation of the wild type stock, Emerson a. Fourteen of these were obtained by Professor Catcheside and the remainder, designated by the prefix 'P', by the author. The other new mutants were obtained following u.v. irradiation of the prototrophic stock, Y8743, by Professor Catcheside at Cambridge some years ago or, of al 38a by Dr. J.R.S. Fincham at Leicester. All the mutants isolated after u.v. irradiation of the Emerson a stock were conidiate and fertile, and readily formed a heterocaryon with an

Table I

Isolation number	Locus number	Linkage group
35203	ad-3	I
30300	arg-3	"
K74	his-2	"
K57	his-3	"
15,300	al-2	"
27,947	arg-5	II
A30 and A36	tryp-3	"
44206t	ad-4	III
33757, 4637T	leu-1	"
H263	pyr-1	IV
37301	pyr-3	"
5531	pan-1	IV
Y2198	tryp-4	"
Y8743c	col-1	"
C102	cot	"
C141	his-4	"
33933	lys-1	V
3254	ad-1	VI
51602t	rib-1	"
75001	tryp-2	"
S15172	sfo	VII

arginine mutant, K132 (arg-5), isolated after u.v. irradiation of the same wild type strain. Most of the other new mutants and the stocks of Beadle's mutants were relatively infertile and none formed a heterocaryotic growth on minimal medium when tested with K132. The reason for this was, presumably, genetically determined incompatibility reactions between K132 and the methionine mutants. Garnjobst (1953, 1955) and Holloway (1955) have demonstrated that the ability of two strains to form a heterocaryon is controlled by a number of genes. Unless these genes are present in the correct combinations, fusion of the hyphae is followed by a protoplasmic incompatibility reaction which prevents heterocaryon formation (Garnjobst and Wilson, 1956). The incompatible stocks were therefore backcrossed, in some cases for three to four generations, to wild type (Emerson a and A) and cultures selected which were conidiate, fertile and heterocaryon-compatible with the test mutant, K132.

Other mutants used as marker stocks were from Professor Catcheside's collection and are listed in Table I. Strain numbers without letter prefixes refer

to mutants in the original Beadle and Tatum (1945) numbering system. Capital letters preceding mutant isolation numbers refer to the original source as follows: A, Professor M. Ahmad; C, California Institute of Technology; E, Dr. Sterling Emerson; H, Dr. Frank Hungate; K, Professor D.G. Catcheside; L, Dr. J.R.S. Fincham; S, Stanford University since 1945 and Y, Yale University.

Media

The standard minimal medium used, which is a synthetic medium failing to support satisfactory growth of a prototrophic strain of Neurospora if any single ingredient is omitted, was that of Vogel (1955). The storage medium consists of:

Sodium citrate ($Na_3C_6H_5O_7.2H_2O$)	127 g.
Potassium dihydrogen phosphate (KH_2PO_4.anhydrous)	250 g.
Ammonium nitrate (NH_4NO_3.anhydrous)	100 g.
Magnesium sulphate ($MgSO_4.7H_2O$)	10 g.
Calcium chloride ($CaCl_2.6H_2O$)	7.5 g.
Biotin	0.25 mg.

Trace element solution	5 ml.
Water	770 ml.
Chloroform (preservative)	2 ml.

This was used as required to make the minimal medium by dilution fifty times with glass distilled water and addition of sucrose to give a 2% concentration. When necessary the medium was solidified by 1.5% agar (Ionagar II supplied by L. Light and Co.). If it was desirable to induce a colonial growth of Neurospora either from ascospores or conidia the 2% sucrose was substituted by 0.5% sorbose and 0.1% sucrose unless stated otherwise. Tatum, Barratt and Cutter (1949) showed that this was a satisfactory way of inducing colonial paramorphism.

Crosses were made on Westergaard's medium (Westergaard and Mitchell, 1947) which differs from Vogel's medium in that potassium nitrate replaces ammonium nitrate as the nitrogen source. The pH of this medium was adjusted to 6.5. In some cases where pyrimidine stocks were used as markers and partial or complete sterility was observed a modification of Westergaard's

medium (Suyama, Woodward and Sarackek, 1959) was used in which the pyrimidine supplement, cytidylic acid, was increased to 50 mg./100 ml. and the sucrose concentration lowered to 0.2%.

The appropriate supplements were added to the minimal medium at the following rates:

L-arginine monohydrochloride	400 mg./litre
Choline	2.4 " "
+DL-allo-cystathionine	500 " "
Cysteic acid	200 " "
L-cysteine	200 " "
Cytidylic acid	100 " "
L-histidine monohydrochloride	300 " "
DL-homocysteine	200 " "
DL-methionine	250 " "
Pantothenic acid (calcium salt)	10 " "
Para-amino-benzoic acid	0.01 " "

All media were sterilised by autoclaving at 10 lbs per square inch for 20 minutes. The supplements were added to the medium before autoclaving except in the cases of cystathionine and homocysteine. These were

sterilised by filtration of the solutions which were then added aseptically to sterilised basal medium.

General Methods

Unless stated otherwise all cultures were incubated at 25°C and stored at 5°C. Ascospore germination was stimulated by heat treatment for 50 minutes in an oven at 56°C.

Tests for precursor utilisation were carried out on appropriately supplemented plates of Vogel's sorbose medium.

Heterocaryon tests were made by superimposing the conidial components on plates of minimal sorbose medium. Originally the conidia were transferred as dry inoculations but later by adding aqueous suspensions dropwise from a Pasteur pipette.

Preliminary linkage data for biochemical mutants were obtained from counts of wild type and mutant ascospores germinated on plates of minimal sorbose medium. The occurrence of 25% of wild types indicates independent segregation of two mutants i.e. 50% recombination. Less

than 25% of wild types denotes linkage of the two.

Confirmation of linkage was sought from ordered tetrads dissected according to the technique described by Beadle and Tatum (1945).

Isolation of New Mutants

An aqueous suspension of conidia contained in an uncovered 2 inch Petri plate was gently shaken beneath an ultraviolet lamp (a low pressure mercury vapour burner from Thermal Syndicate Ltd.) for thirty seconds. Samples of the irradiated suspension were inoculated into incubation tubes (29 x 200 mm.).each containing 25 ml. of liquid minimal medium. These tubes were kept in the dark for an hour to prevent photoreactivation (Kelner, 1949) and then incubated under forced aeration in a constant temperature bath at 25°C. Sterile air from a compressor pump (output 1650 c.c. per minute at 15 cms. mercury) fitted with an air filter was bubbled through the incubation tubes via a glass manifold which enabled four tubes to be aerated simultaneously. The rate of flow of air was controlled by means of screw clips on the rubber tubing connecting each incubation tube to the manifold. The air entered the tubes through a Pasteur pipette, the tip of which only just cleared the bottom of the incubation tube so that thorough agitation of the conidial suspension was

ensured.

The suspensions were passed through cotton wool filters (Catcheside, 1954) after approximately twelve hours incubation by which time the medium had become denser in appearance due to the germination and growth of the wild type conidia. The filter retains the mycelium of the germinated conidia and allows the majority of the ungerminated conidia to pass through. This filtration was repeated three times per day until there were no visible signs of growth within the tubes. This was after approximately 72 hours of incubation. A final filtration of the conidial suspensions was made before plating out in sorbose medium supplemented with methionine and after 24 to 48 hours all the resultant colonies were transferred to slopes of Vogel's medium similarly supplemented. The aconidiate isolates were discarded and the remainder tested for growth on minimal medium. The mutants kept made no or very little growth on minimal medium (i.e. they were 'non-leaky' mutants) but good growth on medium supplemented with methionine.

Cysteine was added to two of the incubation tubes

Table II

Experiment number	Number of incubation tubes	Supplement in incubation tube	Supplement in plating medium	Total number of isolates	Number of aconidiate isolates	Number of isolates tested	Mutants obtained
I	2	-	methionine	185	33	152	61
II	1	-	sulphite	10	3	7	0
	1	thiosulphate	cysteine	25	5	20	0
	2	cysteine	methionine	610	75	535	88
III	1	-	sulphite	36	5	31	0
	1	thiosulphate	cysteine	32	3	29	0
						Total	149

in Experiment II (see Table II) for Experiment I yielded a preponderance of cysteine mutants. This method was also modified, by appropriate supplementation of the media, to select for classes of mutants not represented in the yield from the unselective techniques. In an attempt to isolate sulphite and cysteine mutants irradiated conidial suspensions were incubated in minimal (sulphate) and in thiosulphate medium and plated in sorbose medium supplemented with sulphite and cysteine respectively. This method was repeated but both experiments failed to yield any non-leaky sulphite or cysteine mutants giving good growth on supplemented medium.

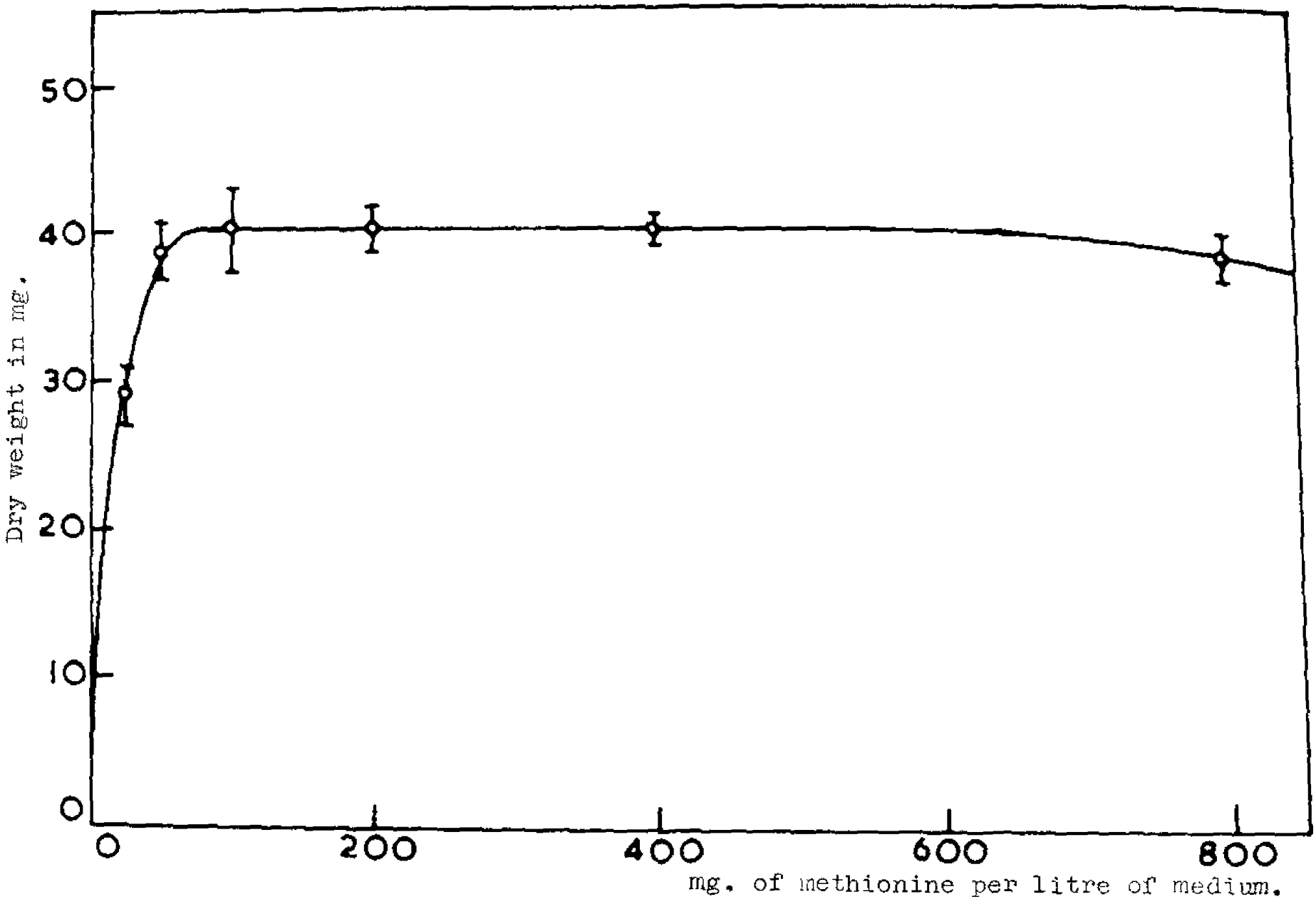
The yields of mutants obtained by this filtration technique are listed in Table II.

Growth Assays

At the beginning of the work a number of methionine stocks reverted for they were found to be capable of growth on minimal medium. A possible explanation was back or suppressor mutation followed by selection through growing the stocks on insufficiently supplemented medium. It was, therefore, decided to determine the optimum concentration of methionine for maximal growth as found by dry weight assays of mycelial pads after four days growth in liquid medium at 25°C.

Portions (20 ml.) of Vogel's medium supplemented with methionine at varying concentrations were pipetted into 250 ml. conical flasks. After autoclaving the flasks were inoculated with one drop of conidial suspension. To keep the mycelium submerged and to prevent conidiation the flasks were swirled three times a day until the harvesting of the mycelium after 96 hours of growth. The mycelial pads were washed with distilled water and the excess water removed by filter paper before drying the pads for five hours in an oven at 80°C. The pads were quickly transferred to a vacuum desiccator and

Fig 1 Mean dry weights of yields plotted against concentration of methionine supplement.



weighed when cool.

Preliminary experiments with mutants 36104 (*me*-3), 38706 (*me*-1) and K22 (*me*-5) indicated that increasing the methionine concentration above 100 mg. per litre did not increase the dry weight yield.

Using mutant K22 the range of methionine concentrations was extended to include from 25 to 800 mg. per litre and four replicates were used for each concentration. The results listed in Table III confirmed the earlier indication and are represented graphically in Fig. I.

For mutant K22 there is, apparently, no significant change in mycelial yield when the methionine concentration is varied between 50 and 800 mg. per litre. The routine concentration subsequently used was 250 mg. per litre, but for storage stocks this concentration was doubled.

Table III

Concentration of methionine in mg/l.	Dry weight of yield (mgm) (x)	Mean dry weight ($\bar{x}$)	Variance $\frac{\Sigma(x-\bar{x})^2}{5}$	Standard deviation $\sqrt{\text{variance}}$
25.0	29.9 27.4 28.5 27.3 32.1	29.04	$\left(\frac{15.1120}{5}\right)$ = 3.02240	1.738
50.0	35.1 38.6 39.8 39.9 39.4	38.56	$\left(\frac{16.1900}{5}\right)$ = 3.2580	1.775
100.0	42.4 40.8 34.8 43.8 39.3	40.22	$\left(\frac{48.1280}{5}\right)$ = 9.6256	3.102*
200.0	42.1 40.0 37.3 40.1 40.5	40.0	$\frac{(11.96)}{5}$ = 2.392	1.548
400.0	40.2 41.8 40.1 39.4 38.7	40.04	$\frac{(5.3321)}{5}$ = 1.0665	1.023
800.0	39.6 37.7 38.1 39.1 36.7	38.12	$\frac{(3.9880)}{5}$ = 0.7856	0.886

*This large standard deviation is due to the one low value obtained, namely 34.8.

A. Physiological aspects of methionine mutants

I. BIOSYNTHESIS

Introduction

At the present time methionine mutants may be placed in one of four groups on the basis of precursor utilisation. One group specifically requires methionine for growth, another can utilise either methionine or homocysteine, a third makes use of both of these and cystathionine and the fourth group any of the above in addition to cysteine. This suggests that cysteine, cystathionine and homocysteine are intermediates in methionine synthesis in Neurospora crassa and that they are produced in the above sequence (Horowitz, 1947). Further evidence for cystathionine as an intermediate results from the discovery (Horowitz, 1947) that the homocysteine mutant, H98, accumulates cystathionine in the mycelium whereas other mutants, blocked at a preceding step, can utilise cystathionine to satisfy their methionine requirement. The recent identification of the enzymes cystathionase I and II by Fischer (1957) with

the demonstration that cystathionase I is absent in a cystathionine mutant, 4894, whilst the homocysteine mutant, H98, lacks cystathionase II confirms the indications of the circumstantial evidence.

It seems likely that the carbon chains of both threonine and methionine are derived from homoserine, for one mutant strain (51504) which requires homoserine will grow on mixtures of threonine and methionine, threonine and homocysteine or threonine and cystathionine (Teas, Horowitz and Fling, 1948). Cystathionine formation probably results from the condensation of cysteine and homoserine (Fling and Horowitz, 1951).

Intermediates between homocysteine and methionine have not been found (Emerson, 1950). Three classes of mutants, each of which blocks the methylation of homocysteine, are distinguished by Zalokar (1950) on physiological grounds based on selenite reduction. Zalokar is also able to place these mutants into three groups, summarised in Table IV, according to the response of the double sulphanilamide, methionine mutant (sfo, me) to methionine and the antagonism by sulphanilamide (SA)

or threonine.

Table IV

Group	Mutants	Response of double mutant to methionine
I	38706 44704 47806	Will not grow in the presence of methionine at any concentration except in the presence of SA or threonine.
II	37603	Grows on low concentrations (2×10^{-5}M) but on high concentrations (2×10^{-3}M) only in the presence of SA or threonine.
III	32213 35809 48003	Initial growth on low and high concentrations but persistent growth only in the presence of SA or threonine.

Members of group I reduce selenite in the presence of additional methionine. Groups II and III fail to reduce selenite whilst a mutant 36102, not listed above, is always able to reduce selenite. A further differentiation between groups II and III is possible for mutant 37603 is able to utilise either methionine or choline as a sole supplement.

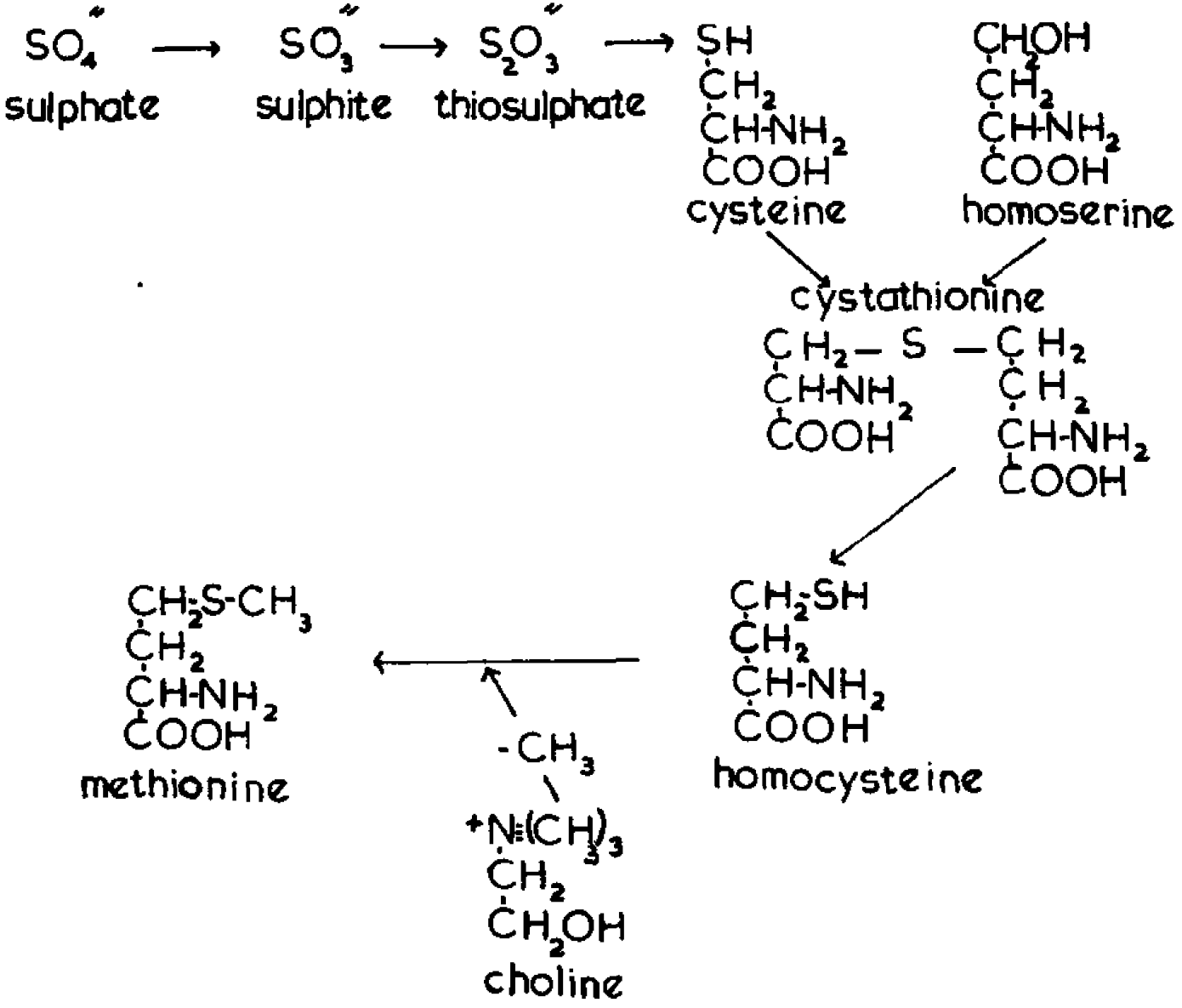
The evidence, therefore, indicates that four different

genetically controlled reactions are involved in the methylation of homocysteine.

That the methyl group of methionine comes from choline is further indicated by the ability of two choline mutants to utilise methionine as a partial replacement for choline (Horowitz and Beadle, 1943; Horowitz, Bonner and Houlahan, 1945; Horowitz, 1946).

The initial stages of methionine synthesis which result in the formation of cysteine from inorganic sulphate are comparatively obscure. Early work using Ophiostoma multiannulatum (Fries, 1946) indicated an inorganic reduction of sulphate via sulphite and thiosulphate to cysteine. In Neurospora the data of Phinney (1948) suggest the coupling of sulphate to an organic compound, possibly alanine, to give cysteic acid and the reduction to cysteine via cysteine sulphinic acid. Horowitz (1955) does not confirm that cysteine sulphinic acid can replace thiosulphate but finds that it has the same growth promoting activity as the further oxidised cysteic acid and, therefore, suggests that the initial steps of the pathway involve only inorganic ions

Fig 11 Outline of methionine synthesis.



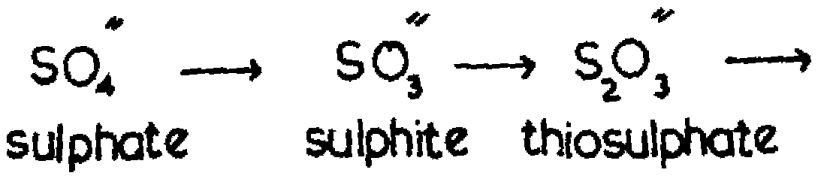
until the sulphur has been reduced to the state of thiosulphate. This is substantiated by studies on a variety of microorganisms including Escherichia coli (Lampen, Roepke and Jones, 1947); Aspergillus nidulans (Hockenhull, 1949); Aspergillus niger (Steinberg, 1941) and Salmonella typhimurium (Clowes, 1958). However, Shepherd (1956) has isolated certain mutants of Aspergillus nidulans which are incapable of growth on sulphite and, in some instances on thiosulphate, but which are able to utilise either cysteic acid or cysteine sulphinic acid. He, therefore, postulates alternative organic and inorganic pathways of sulphur reduction in which either serine, pyruvate or cysteic acid could act as carbon sources.

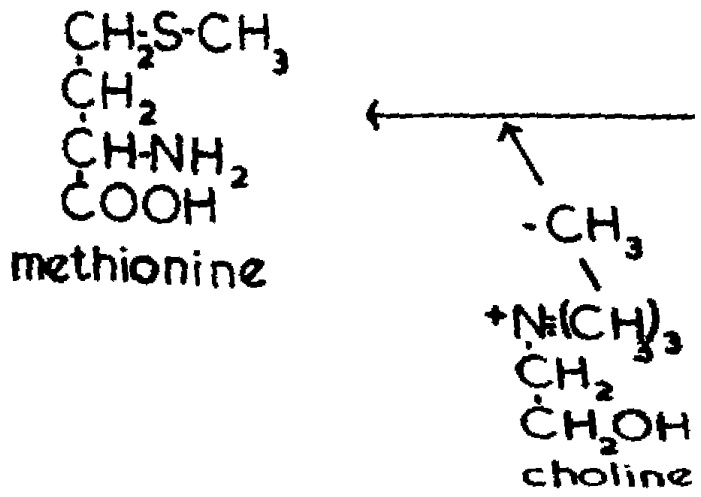
A summary of the proposed synthetic pathway is represented diagramatically in Fig. II.

Experimental

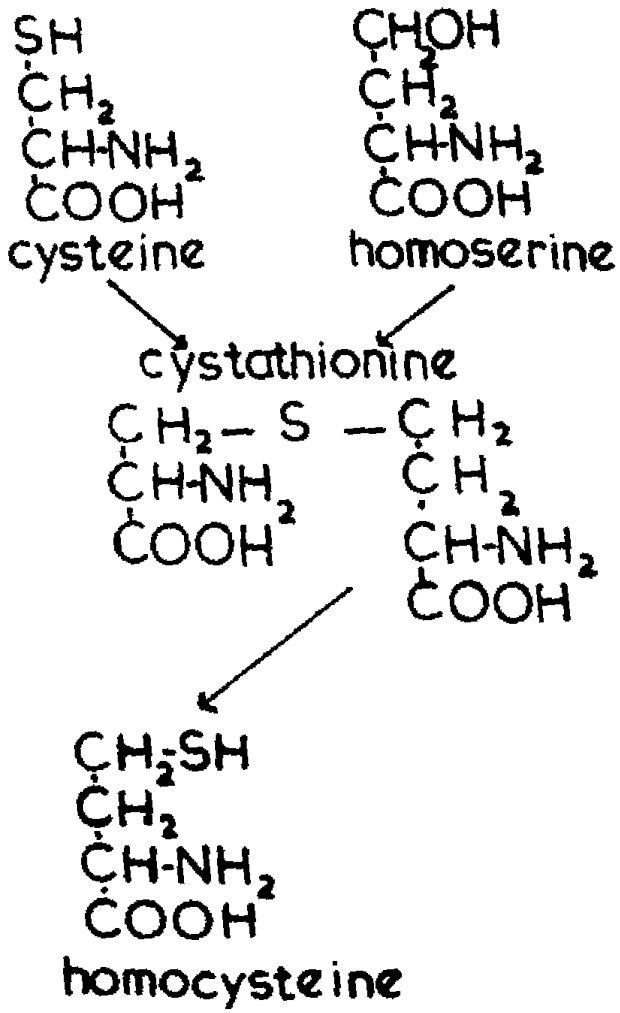
All the methionine mutants were tested to determine which of the reported intermediates would support their growth. Cysteine sulphinic acid was not available but all cysteine mutants were tested for their ability to

Fig 11 Outline of methionine





synthesis.


SH
CH2
CH-NH2
COOH
cysteine
CH2OH
CH2
CH-NH2
COOH
homoserine
cystathionine
CH2 – S – CH2
CH-NH2
COOH
CH2
CH-NH2
COOH
CH2-SH
CH2
CH-NH2
COOH
homocysteine

until the sulphur has been reduced to the state of thiosulphate. This is substantiated by studies on a variety of microorganisms including Escherichia coli (Lampen, Roepke and Jones, 1947); Aspergillus nidulans (Hockenhull, 1949); Aspergillus niger (Steinberg, 1941) and Salmonella typhimurium (Clowes, 1958). However, Shepherd (1956) has isolated certain mutants of Aspergillus nidulans which are incapable of growth on sulphite and, in some instances on thiosulphate, but which are able to utilise either cysteic acid or cysteine sulphinic acid. He, therefore, postulates alternative organic and inorganic pathways of sulphur reduction in which either serine, pyruvate or cysteic acid could act as carbon sources.

A summary of the proposed synthetic pathway is represented diagramatically in Fig. II.

Experimental

All the methionine mutants were tested to determine which of the reported intermediates would support their growth. Cysteine sulphinic acid was not available but all cysteine mutants were tested for their ability to

Isolation numbers of mutants referred to in Table V.

a. 39816, 38401, 84605, K7, K8, K36, L6, P1, P7, P8, P9, P10, P11, P14, P15, P16, P17, P18, P19, P20, P21, P22, P25, P26, P27, P28, P29, P30, P31, P32, P33, P34, P35, P36, P37, P38, P39, P40, P41, P42, P43, P44, P45, P46, P47, P48, P49, P50, P51, P52, P54, P55, P56, P57, P59, P60, P82, P120, P121, P128, P160, P170.

b. 9666, 36104, 39103, 86304, K22, K48, K55, K62, K65, K79, L97, P5, P12, P64, P71, P91, P100, P102, P111, P141, P150, P155, P167, P171.

c. H98, 48004, K5, K18, K23, K43, K44, K49, K76, K86, K98, P2, P24, P71, P72, P73, P78, P80, P81, P85, P88, P95, P99, P101, P107, P110, P113, P118, P126, P131, P132, P133, P140, P143, P145, P147, P151, P152, P153, P154, P159, P162, P169, P174.

d. 35599, 35809, 38706, K59, K66, L134, P3, P4, P6, P13, P23, P53, P58, P61, P63, P65, P67, P68, P70, P74, P76, P77, P83, P84, P86, P89, P93, P94, P96, P97, P98, P105, P108, P112, P114, P117, P119, P122, P123, P125, P127, P129, P134, P135, P136, P138, P144, P148, P149, P156, P158, P165, P168, P175.

utilise cysteic acid. All mutants were tested on homocystine and those blocked between homocysteine and methionine on media supplemented with choline and para-aminobenzoic acid.

The sulphite and thiosulphate media were made by replacing the sulphate of the stock medium by equivalent amounts of sulphite and thiosulphate respectively.

The tests were carried out as stated in the general methods using medium supplemented at the usual rates.

Results

Sulphite, homocystine, choline and para-aminobenzoic acid did not support the growth of any of the mutants. Mutant P120 (*cys*-7) made a very slight growth on cysteic acid. The remainder of the results are given in Table V.

Table V

Number of Mutants	Growth on Methionine Precursors			
	thiosulphate	cysteine	cystathionine	homocysteine
62[a]	+	+	+	+
0	0	+	+	+
24[b]	0	0	+	+
44[c]	0	0	0	+
54[d]	0	0	0	0

+ = growth 0 = no growth

II. HETEROCARYON COMPLEMENTATION

Introduction

Two strains of Neurospora crassa of the same mating type may undergo a vegetative fusion to form a heterocaryon (dicaryon). This is not always the case, for incompatibility factors have been shown to prevent the establishment of the heterocaryotic mycelium (Garnjobst, 1953, 55; Holloway, 1955). If two compatible mutants involved in heterocaryon formation are recessive and not allelic they form a phenotypically wild type (prototrophic) heterocaryon. Conversely, if the mutants are defective at the same locus the formation of a prototrophic heterocaryon would be unexpected. This test of non-allelism is comparable with the phenotypically wild type test shown by a diploid heterozygous for two recessive non-allelic mutant genes. By the heterocaryon tests mutants can be so grouped that members of a group will not form prototrophic heterocaryons with members of their own group but do so with all members of all other groups. Using these theoretical considerations

it is possible to differentiate between allelic and non-allelic mutants blocked between the same precursors.

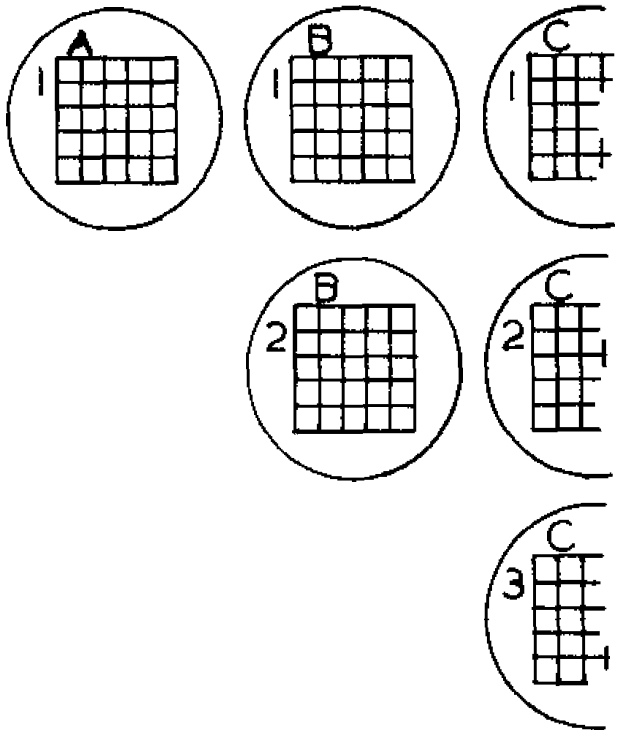
Experimental

All the methionine mutants were heterocaryon compatible with an arginine mutant, K132. It was, therefore, argued that they should be compatible with each other.

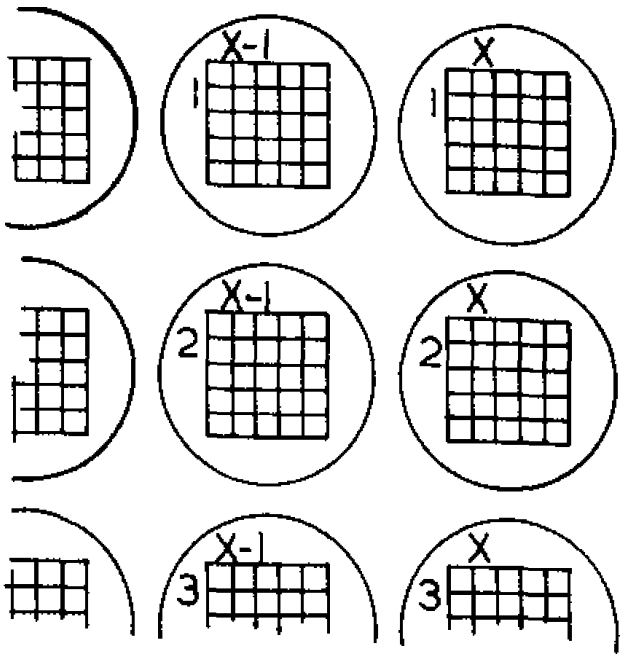
By means of the heterocaryon test all the mutants blocked between the same precursors were grouped so that members within a group did not complement most other members of their group.

The presence of complementation was sought in all the $\frac{n(n-1)}{2}$ possible pairs of combinations within a group of n mutants. The method used (Catcheside, personal communication) involved the inoculation of plates of minimal sorbose medium with an aqueous suspension of conidia from a finely drawn out Pasteur pipette. The drop-wise inoculation was controlled by manipulation of a rubber teat. The base of each Petri plate was marked out in a grid of $\frac{1}{2}$ inch squares

Fig III



Orientation of Petri plates for intra-group heterocaryon tests.


X-1
1
X
1
X-1
2
X
2
X-1
3
X
3
X
N

arranged in five rows of five so that twenty-five pairwise combinations of mutants were tested on each plate. The orientation of the plates was facilitated by labelling one side of the grid with a letter and the adjacent one with a number. The plates were aligned as shown in Fig. III so that in turn each first component was taken rapidly along the appropriate horizontal row and one drop put centrally in each square of the row. The water of the inocula was allowed to become absorbed into the medium before the second components were added in a similar fashion along the vertical rows.

In this way it was possible to make all the intra-group heterocaryon tests for any one group in a day. Formation of a prototrophic heterocaryotic growth was easily detected one to four days after inoculation. Positive results were re-tested using one plate for each test together with the control tests of the components. All the tests were performed at least three times and were found to be consistently reproducible.

Results

The results are summarised in Table VI and the

Table VI

Biochemically "simplest" sulphur requirement	Locus	Previously reported mutants	Number of mutants studied	Inter-allelic complementation
thiosulphate	cys-1	84605	1	-
	cys-2	38401	24	-
	cys-3	-	25	-
	cys-4	-	9	-
	cys-7	-	1	-
	cys-8	-	1	-
	me-4	39816	1	-
cystathionine	me-3	36104	2	-
	me-5	9666, 86304	15	-
	me-7	39103	7	-
homocysteine	me-2	H98, 48004	44	+
methionine	me-1	38706	14	-
	me-6	35809	4	-
	me-8	possibly 36102	35	+
	me-9	possibly 36102	1	-

+ = present - = absent

isolation numbers of the mutants at each locus listed in the descriptive index of mutants (page 65).

Complementation within the group of methionine mutants at locus me-8 was weak and restricted to two combinations as a result of which the mutants are divisible into three groups A, B and C. Members of group A show no complementation with members of either group B or group C but group B and C members complement each other. Figure IVa represents these results in a two dimensional pattern of reaction and Figure IVb is a one dimensional interpretation in which each mutant exerts a given range of influence. These ranges of defective action are so drawn that overlapping ones occur where the heterocaryons do not grow on minimal medium and non-overlapping ranges where complementation results in prototrophic growth of the heterocaryons.

The pattern of complementation at the homocysteine locus, me-2, is more complex but can be represented as shown in Figure V.

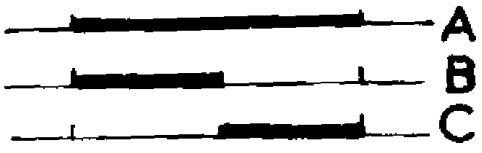
The distribution of the me-2 mutants amongst the complementation classes is shown in Table VII.

Interallelic Complementation Charts

Locus me-8

	B	C
A	○	○
B	●	
C		

a).



	Number in each class
A	32
B	2
C	1

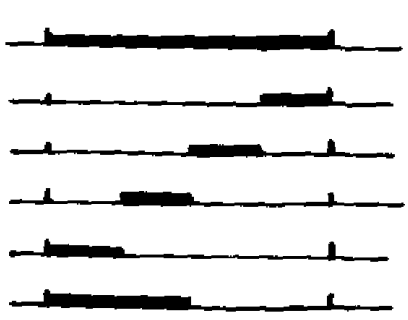
b)

Fig IV

Locus me-2

	B	C	D	E	F
A	○	○	○	○	○
B	●	●	●	●	
C	●	●	●		
D	●	○			
E	○				
F					

a).



	Number in each class
A	32
B	1
C	1
D	2
E	4
F	4

b)

Fig V

○ no complementation

● complementation

Table VII

Group A	K5, K18, K43, K49, K76, P2, P24, P71, P72, P73, P78, P80, P85, P88, P95, P101, P107, P110, P113, P118, P126, P131, P132, P143, P147, P151, P152, P153, P154, P159, P162, 48004.
B (or C)	P169.
C (or B)	P81.
D (or E)	K44, K98.
E (or D)	P99, P174, H98, K86.
F	K23, P133, P140, P145.

The complementation map drawn in Figure V is only one of a number of possibilities. The presence of group F whose range of defective action includes those of groups D and E enables the latter two groups to be allocated to adjacent sites. The absence of other overlapping groups makes it impossible to decide whether mutant P81 or P169 represents group B and, similarly, whether P99 or K44 represents group D. Furthermore, it is conceivable that groups B and C

Table VIII

Groups	F				E				D		C	B
Isolation Numbers	P133	P140	P145	K23	P99	P174	K86	H98	K44	K98	P81	P169
P169	1	1	1	1	1	1	1	1	1	2	4	-
P81	1	1	1	2	1	1	1	1	2	3	-	
K98	-	-	-	-	4	4	4	4	-	-		
K44	-	-	-	-	2	3	3	4	-	-		

Chart of time (in days) taken for inter-allelic heterocaryons to become visible.

are not adjacent but separated by the groups D and E.

Woodward, Partridge and Giles (1958) found that the greater the distance between two ranges ('cistrons') as represented on the linear map, the more vigorous was the heterocaryotic growth. More recently Woodward (1959) has confirmed this indication by enzyme assays on the in vitro complementation at the ad-4 locus. He found a steady increase of enzyme activity with increasing map distance up to a separation of about four "cistrons" after which no further significant increase in activity is observed. If this were the general rule then P169 and P81 must be very close together for they complement each other but weakly, P169 could be assumed to represent group B and P99 group E. Mutant P169 forms heterocaryons with group D more readily than does P81, and P99 forms heterocaryons with group B and C more readily than K44. The figures in Table VIII indicate the days taken for the prototrophic inter-allelic heterocaryons to become visible. The mutants are arranged such that those giving the most vigorous prototrophic heterocaryons are the most widely separated.

It is noticeable that of the two group D mutants K44 gives better complementation than does K98. This is true for four stocks of K98 obtained as single spore isolations from a back cross to wild type.

Discussion

The results of the precursor utilisation tests are consistent with the present outline of the pathway of methionine synthesis, although it is surprising that no sulphite and cysteine dependent mutants were obtained. It is conceivable that traces of sulphite and sulphide in the incubation medium are able to support the growth of these mutants so that they are lost on filtration. None of the cysteine mutants of Neurospora crassa tested by Fischer (Emerson, 1955) were blocked in the synthesis of the carbon skeleton of cysteine for all were able to utilise sulphide or elemental sulphur which is rapidly reduced to sulphide.

Large numbers of mutants able to utilise thiosulphate but not sulphite were obtained and on the basis of heterocaryon tests these mutants were divisible into seven groups of which probably only three have been

reported previously. If the early stages of methionine synthesis can be represented by a simple pathway of reduction the presence of seven blocks between sulphite and thiosulphate would appear excessive. One possibility is the presence of traces of sulphide in the thiosulphate medium sufficient to support the growth of those mutants unable to reduce thiosulphate to sulphide. The sulphide could be present as an impurity in the magnesium thiosulphate or it could result from a decomposition occurring during autoclaving although this seems improbable. In an attempt to differentiate between mutants able to utilise thiosulphate and possible cysteine mutants growing on traces of sulphide, thiosulphate medium was made by adding non-autoclaved sterile sodium thiosulphate (A.R.) aseptically to autoclaved minimal sorbose medium to give a concentration of only 10 µg. of thiosulphate sulphur per ml.. Representatives of five of the seven groups of mutants made good growth on this medium but two previously reported thiosulphate mutants, 39816 (me-4) and 84605 (cys-1) made only a little growth. Stadler

(1956) also found that cys-1 mutants responded much less to thiosulphate than do cys-2 members and this may be a situation comparable to the one found by Clowes (1958) for Salmonella typhimurium where the cys-B mutants require 1 mg. of thiosulphate sulphur per ml. but the cys-E mutants only 10 µg. per ml.. The available evidence does indicate, therefore, that in Neurospora crassa there are seven different genetic blocks between sulphite and thiosulphate.

All the cystathionine mutants fell within the three previously reported groups. Recently an enzyme, cystathionase I, which cleaves cystathionine to yield cysteine, has been isolated by Fischer (1957). A cystathionine mutant, 4894, which is probably an allele of 39103 (me-7), lacked cystathionase I except in the presence of a suppressor gene. Fischer gives evidence for a reversible transulphuration pathway between cysteine and methionine and it will be of interest to establish the relation of the three genetic blocks, between cysteine and cystathionine, to the enzyme cystathionase I and the condensation of cysteine and homoserine to yield cystathionine. Recently

evidence for three unlinked loci functioning in the synthesis of tyrosinase has been obtained (Horowitz et al., 1959). Alleles at one locus determine different forms of tyrosinase whilst mutant alleles of the other two loci are characterised by their lack of the enzyme. This three gene system concerned in cystathionase I production might be similar in nature to the tyrosinase situation for example ~~alternatively~~ locus, me-7, may function in the synthesis of cystathionase I whilst the other two loci are concerned with accessory factors.

The homocysteine mutants were all placed in one heterocaryon group. Fischer (1957) has shown that one member (H98) of this group lacks the enzyme cystathionase II which catalyses the in vitro production of homocysteine from cystathionine. The biosynthesis of homocysteine is probably catalysed by one enzyme, cystathionase II, the production of which is associated with a single locus, me-2.

The heterocaryon tests indicate four genetic groups of mutants blocked between homocysteine and methionine none of which are able to utilise choline. Two of these groups (me-1 and me-6) represent two of the classes

distinguished by Zalokar (1950) on physiological grounds and a third of the four groups may be equivalent to Zalokar's physiolcgical class represented by mutant 36102. The presence of as many as four genetic blocks is not unexpected, for the transmethylation of homocysteine could involve a number of methyl acceptor-donors and possibly an energy producing reaction. It would be necessary, however, to assume that these were specific.

The finding of inter-allelic complementation is in agreement with the recent work of a number of authors (Fincham and Pateman, 1957; Giles, Partridge and Nelson, 1957; Catcheside and Overton, 1958; Lacy and Bonner, 1958). In the first two of these studies the analysis has been extended to the enzyme systems concerned and an enzyme activity of up to, but not exceeding, 25 per cent of that of wild type has been found in inter-allelic heterocaryons. This behaviour, which appears to be fairly widespread, is of significance in relation to the mode of gene influence on enzyme formation.

B. Mapping and linkage data

Introduction

The primary purpose of this study was to look for evidence of the clustering of methionine mutants in linkage group IV and of cysteine mutants in group VI. It was known that locus *me*-5 was situated in the right arm of linkage group IV (Barratt *et al.*, 1954) and that two cysteine loci, *cys*-1 and -2, were located very close to each other in linkage group VI (Stadler, 1956). The two other methionine loci previously identified were *me*-3 (36104) in group V and *me*-6 (35809) in group I (Barratt *et al.*, 1954). It was, therefore, decided to assign the previously unlocated mutants to linkage groups by means of preliminary data from random spores. More detailed and accurate information could then be obtained from studies on ordered tetrads.

Theory of Methods

Linkage between two mutants was detected when the frequency of wild type recombinants, amongst the random progeny of a cross involving the two factors in the repulsion phase, was significantly below 25 per cent.

(A frequency of 25 per cent is taken as an indication of 50 per cent recombination for the two recombinant classes must be produced with equal frequency.)

Confirmation of the preliminary indication of linkage was sought in the analysis of data from ordered asci. Such an analysis is also able to provide data for the estimation of recombination frequencies between a gene and either another gene or the centromere, and to establish the position of a locus with respect to the centromere and other markers.

During meiosis the centromeres of the paired chromosomes separate undivided at the anaphase of the first division. Hence, if no cross-over occurs between a gene locus and the centromere the gene segregates with the centromere at the first division. Where a cross-over occurs between the locus and centromere the segregation of that locus occurs at the second division. The frequency with which second division segregation occurs for a particular locus is a measure of the frequency of crossing-over proximal to that locus and hence of the centromere distance.

In a chromosome interval delimited by two genes a single cross-over results in two parental and two recombinant chromatids (i.e. 50 per cent recombination) for only two of the four chromatids are involved in the cross-over. An interval in which an average of one cross-over per ascus occurs is defined as having a map distance of 50 units and this, neglecting the occurrence of multiple cross-overs, is equivalent to either 50 per cent recombination or 100 per cent of second division segregation.

From a cross of Ab x aB three types of ascus compositions are possible neglecting differences due to spore order: Ab, Ab, aB, aB, (parental ditype); Ab, ab, aB, AB, (tetratype) and AB, AB, ab, ab, (non-parental ditype). These are abbreviated to PD, T, and NPD respectively throughout the text. If factors A and B are on the same chromosome a PD ascus represents either the non cross-over or the two-strand double cross-over class and a T ascus would result from either a single cross-over or a three-strand double cross-over between the two loci. The production of a NPD ascus would be evidence of a

four-strand double cross-over and this class is, therefore, expected to be rare. Alternatively if the two loci are on separate chromosomes PD and NPD asci will result when both loci segregate at the first division and these two classes are expected to occur with equal frequency. A significant excess of PD over NPD asci, therefore, is only possible if the two loci are on the same chromosome. The ratio of NPD to T can not exceed 1:4 if the two genes are linked for NPD segregations of linked genes can result only from $\frac{1}{4}$ of double exchanges + $\frac{1}{8}$ of triples + $\frac{3}{16}$ of quadruples + $\frac{5}{32}$ of quintuples etc.. If the genes are on separate chromosomes there is no upper limit to the NPD to T ratio.

From the analysis of spore arrangements in the asci, particularly where the unmarked intervals are not too long, it is possible to deduce the cross-overs which must have taken place at each meiosis for any given order of genes and centromere. Such an analysis (Whitehouse, 1942) can be made for each possible arrangement of the markers and the one requiring the simplest cross-over patterns is the most likely.

Initial experiments were designed to detect linkage between cysteine mutants and markers in linkage group VI and methionine mutants and markers in group IV. Where no evidence of linkage was obtained, ordered asci were dissected from a cross of the mutant to a third marker in another linkage group and the centromere distance estimated as half of the precentage of second division segregation frequency. (The data from these tetrads also served to detect linkage, if present, between the mutant and the third marker.) Where the centromere distance was fairly small mapping proved relatively simple and crosses were made between the unlocated mutant and markers close to the centromeres of the other five linkage groups. Preliminary indication of linkage was confirmed by analysis of ordered asci. Where segregation of the mutant locus was independent of the centromere, location of the mutant was more tedious. Crosses were made, wherever possible, to markers in both arms of each chromosome and evidence of linkage sought amongst data from the resulting spores.

The allelism of members of a heterocaryon group

was confirmed by the absence of wild type recombinants amongst the progenies of intra-group crosses. Conversely, the non-allelism of mutants from different heterocaryon groups was shown by the presence of wild type recombinants amongst the progenies of inter-group crosses, wherever examined, and usually by the assignment of the mutants to different chromosome regions.

Experimental Details

The frequency of wild type formation in the progeny from a cross involving two biochemical markers was estimated from counts of prototrophic and auxotrophic asco⁀spores germinated on plates of minimal sorbose medium. In practice it was found that this method can yield erroneous results for certain stocks were found to carry deleterious factors affecting viability. Such factors either prevented the growth of the germinated ascospore or, more specifically, prevented the growth in the presence of sorbose. In either case the effect was to decrease the estimate of wild type frequency and hence give a false indication of linkage.

When outcrossing to wild type failed to remove these

factors data from random spores was obtained by a more reliable method in which the ascospores were germinated on appropriately supplemented plates of sorbose medium and the growing spores were transferred to tubes of similarly supplemented Vogel's medium. The genotype of each isolated culture was then ascertained in the usual way. A significant excess of parental type over recombinant spores was taken as evidence for linkage.

Tetrads were dissected according to the method of Beadle and Tatum (1945) but using mounted nickel-plated sewing needles. The ascospores were transferred singly, on blocks of agar medium, to tubes of supplemented Vogel's medium, but in some cases adjacent pairs of spores were transferred together to the same tube. Unless stated otherwise the data used is from those asci where at least one member of each spore pair germinated.

Results

(i) Mutants blocked between cysteine and cystathionine.

Locus me-5 (represented by mutant K22). This

locus was known to be in the right arm of linkage group IV. In an attempt to ascertain the position of me-5 more precisely, ordered asci were dissected from the cross K22 x Y2198, 5531 (tryp-4, pan-1) which involved two markers in the right arm of group IV thereby increasing the possibility of detecting multiple cross--overs. Amongst 54 asci no recombination was observed between the pan-1 and me-5 loci hence it was impossible to order these two loci with respect to the centromere.

Considering the me-5 and tryp-4 loci, only, the results are tabulated in Table IX according to the segregation classes (Catcheside, 1951) outlined below.

Class I Division I segregation for both loci with genes in parental combination.

II Division I segregation for both loci with genes recombined.

III Division I segregation for locus $\frac{a}{\lambda}$ (me-5) and Division II segregation for b (tryp-4).

IV Division II segregation for locus a (me-5) and Division I segregation for b (tryp-4).

V Division II segregation for both loci with genes in parental combination.

Class VI Division II segregation for both loci with genes recombined.

VII Division II segregation for both loci giving four spore types.

Table IX

Ascus class	I	II	III	IV	V	VI	VII	Total	Division II segregation of *me*-5	Division II segregation of *tryp*-4
Number	24	0	3	9	18	0	0	54	27 (50.0%)	21 (38.9%)

The centromere distances are 19.45 units for *tryp*-4 and 25 units for *me*-5, hence the distance between the two loci, obtained by difference, is 5.55 units. The distance between these two loci calculated as half the percentage of asci in which a cross-over occurs between *me*-5 and *tryp*-4 is $\frac{12 \times 100}{2 \times 54}$ which equals 11.1 units. The difference between these two values is due to the three representatives in class III which show first division segregation of *me*-5 as the result of a two- or four-strand double cross-over between *me*-5 and the centromere.

Locus *me*-5 is distal to *tryp*-4 on the basis of their centromere distances and from consideration of the asco-

spore arrangements, for if it were proximal, the nine members of class IV, instead of the three members of class III, would have to be formed by two- or four-strand double cross-overs with one cross-over between the centromere and me-5 and one between me-5 and tryp-4.

No datumis available for intra-group crosses as they were almost sterile.

Locus me-7 (represented by mutant 39103). Preliminary linkage data suggested that locus me-7 was in linkage group IV for wild type frequencies of approximately 10 per cent were obtained from counts of spores from crosses between mutant 39103 and a number of linkage group IV markers.

Of 23 asci dissected from the cross 39103 x Y2198, 5531, considering only the me-7 and tryp-4 loci, there were 9 NPD, 3 PD and 10 T type asci. Furthermore, locus me-7 showed no second division segregation and it was concluded that me-7 is not in linkage group IV.

Random spores were examined from crosses of 39103 to markers close to the centromeres of the other linkage groups. Only one wild type recombinant was observed amongst 2,000 viable spores from a cross 39103 (me-7)

x 15172 (sfo, in linkage group VII).

Ordered asci were dissected from this cross but as the spores were not very viable the results were based on those asci with a minimum of one of each of three spore pairs germinating. All the fourteen asci dissected showed PD segregation.

Of 44 asci dissected involving locus me-7 all have shown division I segregation of this locus. Locus me-7, therefore, is situated in linkage group VII with a centromere distance not exceeding ($\frac{1}{2}p$, where $(1 - p)^{44} = \frac{1}{20}$), 3.3 units at the probability level of 5 per cent or 4.9 units at the 1 per cent probability level.

No wild type recombinant was observed amongst approximately 3,000 viable spores from an *intra-group* cross.

Locus me-3 (mutant 36104). This locus had previously been shown to be in the right arm of linkage group V. No further datum was obtained.

An *intra-group cross* was not very fertile but no wild type recombinant was observed amongst 300 viable spores.

(ii) Mutants blocked between cystathionine and homocysteine.

Locus me-2 (represented by mutant K23). The mutant K23 had been produced in a stock (Y8743c) carrying col-1, which is in the right arm of linkage group IV, and it was soon found that these loci were closely linked. The classification of 59 ordered asci, from the crosses K23 col-1a x Em A and K23 col-1A x Em a, according to the classes listed on pages 44 and 45, is shown in Table X where me-2 represents locus a and col-1, locus b.

Locus me-2 is proximal to col-1 for otherwise the eight members of class III, assumed to be formed by a single cross-over between the two loci, would result from two- or four-strand double cross-overs with one cross-over between the centromere and col-1 and one between col-1 and me-2. The former assumption is in agreement with the centromere distances. The centromere distance of K23 is 22.9 units and of col-1 is 29.7. The distance between the two loci calculated from the number of cross-overs detected between them is $\frac{9}{2 \times 59} \times 100 =$ 7.6 units.

Table X

Locus b	Ascus classes							Total Number	Division II Segregation		% Recombination frequency
	I	II	III	IV	V	VI	VII		of me-2	of locus b	
pyr-3	3	0	0	5	6	0	1	15	12	7	20.0
tryp-4	28	1	0	4	6	0	1	40	11	7	8.75
pan-1	26	0	3	0	11	0	0	40	11	14	3.75
col-1	24	0	8	0	26	0	1	59	27	35	7.63
his-4	11	0	4	0	9	0	0	24	9	13	8.33
cot	7	0	4	3	6	0	1	21	10	11	19.05

Locus me-2 provided a good example of inter--allelic complementation and inter-allelic crosses were quite fertile yielding abundant ascospores usually with approximately 50 per cent germination. It seemed, therefore, that this locus would be a useful one to study in an attempt to correlate complementation and site maps. Crosses were made between K23 and a number of markers which might be close to the me-2 locus. By analysis of ordered asci dissected from these crosses, it was hoped not only to position locus me-2 with respect to the centromere and the markers, but also to find two suitable markers situated one on either side of the locus.

The analyses of these crosses are shown in Table X. The recombination frequencies listed in the table are calculated as half of the percentage of asci with cross-overs occurring between me-2 and the marker. From consideration of ascospore arrangements locus me-2 is distal to pyr-3 and tryp-4, but proximal to pan-1, col-1, his-4 and cot.

Of a total of 229 asci dissected involving locus me-2 92 (40.2 per cent) showed second division segregation

Table XI

Linkage between various homocysteine mutants and the cystathionine mutant (K22) analysed for heterogeneity

Allele	Mating type of stock	Number of crosses of different stocks of the same allele	Number of samples of each cross	Number of germinating spores from each cross (n)	Number of prototrophs (n)	% Recombination $\left(\frac{2n}{m} \times 100\right)$	P as found by heterogeneity χ^2's: Between samples of same stock	Between different stocks of same allele	Between reciprocal crosses	Between alleles
K5	A	1	4	2374	58	4.87	.95 - .90			.95
	a	1	4	2071	34	3.3	.98 - .94		.01	
K18	A	2	3	408	35	17.1 *2	> .98	.01		
			6	2615	84	6.4	.30 - .20			
K23	a	1	8	2126	152	14.3 *1	< .01			
	A	1	2	1351	36	5.3	.30 - .20			
N98	A	1	1	460	10	4.3				

*1 results discarded and not used in further calculations because of heterogeneity between samples.

*2 results discarded and not used in further calculations because of heterogeneity between crosses.

corresponding to a centromere distance of 20.1 units.

The pan-1 and tryp-4 loci were chosen as suitable markers for the investigation of recombination within locus me-2.

It seemed possible that the loci me-5 and me-2 formed part of the cluster of methionine loci postulated by Horowitz (Demerec, 1955). During the early stages of this study, therefore, crosses were made between mutant K22 (me-5) and a number of homocysteine mutants to determine whether or not these two loci were closely linked. The data were obtained from counts of wild type (recombinant) and mutant ascospores, germinated on minimal sorbose medium, before the difficulty due to viability factors had been encountered. It is now realised that the data (Table XI), obtained in this way, are suspect but the result of deleterious viability factors not preventing germination, would be a decrease in the recombination frequency thereby giving a false indication of the proximity of the loci. The data are also imperfect for two other reasons. First, the recombination frequencies of the different crosses are not homogeneous.

This is due entirely to the two crosses which gave the highest values of recombination for both showed low fertility of the ascospores, and in one case different samples of the same cross were heterogeneous, suggesting some environmentally caused variation of germination. If these two sets of data are eliminated the remaining crosses, which showed better fertility, are homogeneous. Secondly a proportion of the prototrophs were pseudo-wild types (Pittenger, 1954). Indeed 25 out of the 76 which were successfully tested were pseudo-wild types (PWT). The apparent recombination frequency, excluding the heterogeneous data and corrected for the presence of PWTs, is $\frac{222 \times 2 \times 51 \times 100}{8871 \times 76}$ = 3.3 per cent. This value is not consistent with the two loci being adjacent.

The data from inter-allelic crosses are considered fully in Section V.

(iii) Mutants blocked between homocysteine and methionine.

Locus me-1 (represented by mutant 38706). Since Horowitz (personal communication to Professor Catcheside) found that locus me-1 was close to the centromere of

linkage group IV, asci from a cross of 38706 to a mutant H263, at locus pyr-1, situated fairly close to the centromere but in the right arm of linkage group IV, were dissected. The analysis, according to the segregation classes used previously, of the twenty asci dissected is shown in Table XII where pyr-1 is locus b.

Table XII

Ascus class							Total	Division II segregation	
I	II	III	IV	V	VI	VII		of me-1	of pyr-1
16	0	0	2	2	0	0	20	4	2

If the two loci are in opposite arms of the linkage group the two class V members are the result of two-strand double cross-overs with one cross-over between each locus and the centromere. Assuming that there is no chromatid interference and using the data in Table XII the expected frequency of the above event would be only $\frac{4}{20} \times \frac{2}{20} \times \frac{1}{4} \times 100 = 0.5$ per cent. Furthermore, if the loci are in the same chromosome arm with pyr-1 distal to me-1, two occurrences of two- or four-strand doubles with one cross-over

between me-1 and the centromere and one between me-1 and pyr-1 must be postulated to account for the two members of class IV. The simplest interpretation of the data is that pyr-1 and me-1 are in the right arm of linkage group IV with locus me-1 distal to pyr-1, and that the members of class IV result from a single cross-over between the two loci.

Locus me-6. No information was obtained for this locus which had previously been reported to be in the right arm of linkage group I (Barratt et al., 1954).

Locus me-8 (represented by mutants K66 and P61). Preliminary linkage data indicated that me-8 was not in linkage group IV. Asci were dissected from crosses of either K66 or P61 to the linkage group I markers, 35203 (ad-3) and K74 (his-3). Since 16 PD, 12 NPD and 6 T type asci constituted the 34 asci analysed, and only five of 45 asci dissected, involving locus me-8, showed second division segregation of this locus it was concluded that locus me-8 is fairly close to the centromere of a linkage group other than I. Crosses were made of me-8 mutants to markers close to the centromeres of the other

linkage groups and the spore progenies examined. No wild type recombinant was observed amongst approximately 450 random spores from a cross to mutant 44602t (locus ad-4), in the right arm of linkage group III. The analysis of the 24 viable asci dissected from this cross is shown in Table XIII where locus b is ad-4.

Table XIII

Ascus class							Total	Division II segregation	
I	II	III	IV	V	VI	VII		of me-8	of ad-4
23	0	0	0	1	0	0	24	1	1

No recombination was observed between the two loci. The simplest interpretation of the ascospore arrangements is that both loci are in the right arm of linkage group III and that the class V member arose as the result of a single cross-over between the centromere and the more proximal of the two loci.

Locus me-9 (mutant P156). Mutant P156 showed no linkage to mutant 38706 (locus me-1, linkage group IV), for of 185 spores germinated on minimal sorbose medium 50 (54 per cent) were wild type. Examination of the progenies from crosses of mutant P156 to mutants 35809

(locus me-6) and P61 (locus me-8) revealed recombination frequencies of approximately 50 per cent.

The data, therefore, do not permit the allocation of mutant P156 to a linkage group but do confirm that mutant P156 is defective at a locus me-9, distinct from the three other loci (me-1, me-6 and me-8), which are associated with the methylation of homocysteine to methionine.

(iv) Mutants blocked between sulphite and thiosulphate.

Locus me-4 (mutant 39816). Preliminary linkage data from a cross of 39816 to the group VI marker, 51602t (locus rib-1), indicated that locus me-4 was probably in linkage group VI and ordered asci were dissected from this cross. The viability of these asci was poor and the results analysed are based on those tetrads in which a minimum of one of each of three spore pairs was viable. Twelve such asci were dissected and of these 3 showed PD, 3 NPD and 6 T type segregation and five of the twelve asci showed second division segregation for me-4. Of a further six asci dissected from a cross of 39816 to a linkage group I marker, K74 (locus his-3), 2 were NPD and 4 T type in

segregation pattern. The latter four asci showed second division segregation of me-4 and, hence, of a total of 18 asci dissected 9 showed division II segregation of this locus. It was concluded that locus me-4 was not in either linkage group I or VI and that it was not close to a centromere.

Estimates of recombination frequencies based on random spore counts from crosses of 39816 to markers in a number of linkage groups were approximately 20 per cent suggesting that the stocks of this mutant carried deleterious viability factors leading to false indications of linkage. However, a recombination frequency of approximately only 4 per cent was obtained, using the same method, for a cross involving 39816 and the linkage group V mutant, 36104 (locus me-3). Spores were, therefore, germinated on plates of sorbose medium supplemented with methionine and the growing colonies isolated. Only 12 of the 119 colonies isolated were found to be wild type, although if the loci me-4 and me-3 were unlinked a ratio of one prototroph to three auxotrophs would be expected. The observed values of 12 wild types to 107

mutants were tested for deviation from the expected values of 29.75 wild to 89.25 mutant types and a total χ^2, for one degree of freedom, of 13.73 was obtained (*Table XIV*).

Table XIV

Class	Number observed (a)	Number expected (mn)	Deviation (a-mn)	$\chi^2 = \left\{\frac{(a-mn)^2}{mn}\right\}$
Prototrophs	12	29.75	-17.5	10.3
Auxotrophs	107	89.25	+17.5	3.43
Total	119.0	119.00	0.00	13.73

The probability of obtaining the observed χ^2 value by chance is much less than .01 and it is concluded that locus *me*-4 does not recombine independently of locus *me*-3.

The recombination frequency calculated from the number of wild type spores is $\frac{12 \times 2}{119} \times 100 = 21.6$ per cent.

Locus *me*-4, therefore, is probably in linkage group V but confirmation by analysis of data from ordered asci has not yet been sought.

Loci *cys*-1 and *cys*-2. These loci are situated very close to each other and to the centromere in the

and A30 (locus tryp-3), and to the biochemical group I markers, 35809 (me-6) and 35203 (ad-3), were infertile. A cross of P29 to the morphological mutant 15300 (al-2, in linkage group I) was fertile and spores from this cross were germinated on plates of sorbose medium supplemented with methionine. A total of 175 growing ascospores were isolated and tested for growth on minimal medium. The recombinants, either mutant or wild type with respect to both the cys-3 and al-2 markers, totalled 70 as opposed to 105 parental type spores. If the loci were unlinked the two classes should be expected with equal frequency (i.e. 87.5 in each class). The χ^2 value for the deviation from the expected is $2 \times \frac{(17.5)^2}{87.5} = 7.0$ for one degree of freedom. The probability of obtaining such a value by chance is less than 0.01 and the data, therefore, indicate that the two loci are linked.

The recombination frequency between the two loci is $\frac{70}{175} \times 100 = 40$ per cent.

Thus the cys-3 locus is probably in linkage group I but confirmation has not yet been obtained.

Locus cys-4 (represented by P33). Preliminary estimates of linkage between P33 and a linkage group VI

marker 75001 (locus tryp-2) indicated that cys-4 might be in this linkage group. Ordered asci were dissected from this cross but viability was poor and the data are based on asci in which a minimum of one of each of three spore pairs was viable. Of 18 asci dissected 1 showed PD, 1 NPD and 16 T type segregation giving no evidence of linkage and 11 of the 18 asci showed second division segregation for locus cys-4.

Crosses were made to a number of markers and the data from random spore counts are summarised in Table XVI.

Table XVI

Marker			No. of viable spores		
Isolation no.	Locus no.	linkage group	wild type	total	% Recombination
35809	me-6	I	40	146	56
27947	arg-5	II	31	128	48
44602t	ad-4	III	52	238	44
C141	his-4	IV	25	108	46
36104	me-3	V	38	255	30
75001	tryp-2	VI	56	308	36

No progress has been made in the allocation of

locus cys-4 to a linkage group.

Intra-group crosses between mutants K7 and K8 gave 7 prototrophs in approximately 9,000 spores and between P1 and K8, 3 prototrophs in approximately 5,000 spores.

Loci cys-5 and cys-6. Stocks of mutants at these loci were not available and there is no published data concerning the mapping of such mutants.

Locus cys-7 (mutant P120). Amongst 224 random spores, germinated on minimal sorbose medium, from a cross between mutants P120 and L6 (locus cys-2), 36 (16 per cent) wild type recombinants were observed. From this evidence it is concluded that locus cys-7 is not situated close to the cys-1 and -2 loci in linkage group VI.

Locus cys-8 (mutant P160). Amongst 142 random spores, germinated on minimal sorbose medium, from a cross between mutants P160 and 84605 (locus cys-1), 30 (21 per cent) wild type recombinants were observed. It is, therefore, concluded that locus cys-8 does not form part of a linkage group VI cluster.

Discussion

Two loci concerned in the metabolic pathway cysteine $\underline{me}$-5 cystathionine $\underline{me}$-2 homocysteine have been associated with a fairly short region in the right arm of linkage group IV. A third locus ($\underline{me}$-1), which is concerned with the methylation of homocysteine to yield methionine, is situated in the same arm of this linkage group, but much closer to the centromere. Therefore, the linkage group IV cluster, indicated by Horowitz, is not a close one with the genes adjacent to one another.

A second locus ($\underline{me}$-7), concerned in the condensation of cysteine and homoserine to form cystathionine, has been identified in linkage group VII and locus $\underline{me}$-8, mutation of which causes a blockage in methylation of homocysteine, has been allocated to linkage group III. Mutant P156, locus $\underline{me}$-9, also associated with the methylation of homocysteine, has not been identified with any chromosome region but linkage data confirm that mutant P156 is defective at some locus other than $\underline{me}$-1, $\underline{me}$-6 or $\underline{me}$-8. As the loci, $\underline{me}$-3 and $\underline{me}$-6, had been identified

previously, three cystathionine, one homocysteine and three methionine loci can now be associated with particular chromosome regions.

The mapping of the cysteine loci has been much less complete for attention was concentrated on the methionine mutants. Stocks of mutants unable to reduce sulphate to sulphite (locus cys-5) and mutants unable to reduce thiosulphate to sulphide (locus cys-6) have not been available for study. Of the seven groups of cysteine mutants studied, the cys-1 and cys-2 loci had been identified in linkage group VI by Stadler (1956) but no further indications were found for the location of any other mutants in a linkage group VI cluster. The previously reported locus, me-4, is probably in linkage group V and it seems likely that the remaining four of the seven groups of cysteine mutants, all unable to reduce sulphite to thiosulphate, represent loci that have not been reported previously. Evidence has been obtained that locus cys-3 is in linkage group I but the cys-4, cys-7 and cys-8 loci have not been associated with any linkage group.

A summary of the information obtained with respect

to the precursor utilisation and location of the methionine mutants is given in the form of a descriptive index compiled on the lines of that of Barratt et al. (1954). The isolation numbers of the alleles at each locus, based on heterocaryon tests, are included.

Locus designation	Description
cys-4	Linkage group unknown. P33. Utilises thiosulphate; cysteine; cystathionine; homocysteine or methionine but not sulphite. Alleles: K7, K8, P1, P45, P55, P82, P121, P128.
cys-5	Linkage group unknown. 35001. Utilises sulphite; thiosulphate; cysteine; cystathionine; homocysteine or methionine (Phinney, 1948).
cys-6	Linkage group unknown. 86801. Utilises cysteine; cystathionine; homocysteine or methionine but not thiosulphate or sulphite (Phinney, 1948).
cys-7	Linkage group unknown. P120. Utilises thiosulphate; cysteine; cystathionine; homocysteine or methionine but not sulphite.

Locus designation	Description
cys-8	Linkage group unknown. P160. Utilises thiosulphate; cysteine; cystathionine; homocysteine or methionine but not sulphite.
me-1	Linkage group IV, right arm. Order: distal to pyr-1. 38706. Utilises methionine but not homocysteine. Alleles: 35599, K59, L134, P23, P58, P63, P84, P89, P97, P98, P105, P117, P144.
me-2	Linkage group IV, right arm. Order: distal to pyr-3 and tryp-4 but proximal to pan-1, his-4, col-1 and cot. H98. Utilises homocysteine and methionine but not cystathionine. Alleles: 48004, K5, K18, K22, K43, K44, K49, K76, K86, K98, P2, P24, P71, P72, P73, P78, P80, P81, P85, P88, P95, P99, P101, P107, P110, P113, P118, P126, P131,

Locus designation	Description
	P132, P133, P140, P143, P145, P147, P151, P152, P153, P154, P159, P162, P169, P174.
me-3	Linkage group V, right arm (Barratt et al., 1954). 36104. Utilises cystathionine; homocysteine or methionine but not cysteine. Allele: P155.
me-4	Probably linkage group V. 39816. Utilises thiosulphate (at higher concentrations); cysteine; cystathionine; homocysteine or methionine but not sulphite.
me-5	Linkage group IV, right arm. Order: distal to tryp-4. 9666. Utilises cystathionine; homocysteine or methionine but not cysteine. Alleles: 86304, K22, K48, K55, K62, K65, P75, P91, P100, P102, P111, P141, P167, P171.

Locus designation	Description
me-6	Linkage group I, right arm (Barratt et al., 1954). 35809. Utilises methionine but not homocysteine. Alleles: P67, P108, P135.
me-7	Linkage group VII, close to centromere. 39103. Utilises cystathionine, homocysteine or methionine but not cysteine. Alleles: K79, L97, P5, P12, P64, P150. Comment: Probably allelic to 4894 (Fischer, 1957).
me-8	Linkage group III, right arm. P61. Utilises methionine but not homocysteine. Alleles: K66, P3, P4, P6, P13, P53, P65, P68, P70, P74, P76, P77, P83, P86, P93, P94, P96, P112, P114, P119, P122, P123, P125, P127, P129, P134, P136, P138, P148, P149, P158, P165, P168, P175. Comment: Could be allelic with 36102 (Emerson, 1950).

Locus designation	Description
me-9	Linkage group unknown. P156. Utilises methionine but not homocysteine. Comment: Could be allelic with 36102.

C. Intragenic recombination

Introduction

The term "gene" has been used to signify the hereditary determinants which are passed from parents to offspring and which are in some way responsible for the development of the observed characters of an organism. The more precise definition of the gene states that the gene is the smallest unit of inheritance; the ultimate unit of phenotypic difference; and the unit of mutation. The first of these criteria indicated that the gene was indivisible by recombination or chromosome breakage; the second that the gene could be associated with a single primary metabolic function; and the third that it was the smallest part of a chromosome which, when changed, would be replicated in the changed form.

From analysis of the "scute" region of Drosophila melanogaster, Raffel and Muller (1940) concluded that the three definitions of the gene need not be co-extensive. The discovery of recombination between the two physiologically allelic mutants "Star" and "asteroid" in Drosophila

(Lewis, 1945) and, more recently, between alleles at loci in Aspergillus nidulans (Pritchard, 1955); Neurospora crassa (Giles, 1951, 1956; Mitchell, 1955); and yeast (Roman, 1956), suggest that the ability of physiologically allelic mutants to recombine may be the general rule rather than the exception.

Pritchard (1955), studying a number of physiologically allelic adenine mutants of Aspergillus, has been able to place the alleles in a linear order interpreting the locus as comprising a number of sites of mutation separable by crossing-over. Pritchard's analysis was controlled by markers on either side of the adenine locus. In general, recombination within the locus was associated with recombination of the outside markers and, therefore, prototroph formation was explicable as the result of a single cross-over between the defective sites of the locus. Such a cross-over would produce one normal and one doubly mutant chromatid strand and Pritchard was able to recover the doubly mutant recombinant. Other prototrophs, of parental phenotype with respect to the outside markers, could not be interpreted as the products

of single intra-genic cross-overs but were, perhaps, comparable with the prototrophs from crosses of allelic pyridoxin mutants of Neurospora (Mitchell, 1955a, 1955b) formed as the result of what Mitchell termed "aberrant recombination". Mitchell studied aberrant recombination in ordered asci and found that prototroph formation was not accompanied by the formation of the reciprocal double mutant as would be expected if a double cross-over had taken place.

The evidence thus suggests that two processes may be involved in inter-allelic recombination. The first is intra-genic crossing-over demonstrated by Pritchard (1955) and the second is the unidirectional transfer in heterozygotes described in Saccharomyces cerevisiae (Lindegren, 1953; Roman, 1956); in Neurospora crassa (Mitchell, 1955a, 1955b; Case and Giles, 1957) and in Schizosaccharomyces pombe (Leupold, 1957).

Various hypotheses have been developed to describe the possible mechanism of "aberrant recombination". These include the directed mutation or "conversion" hypothesis (Lindegren, 1953; Strickland, 1958) the copy choice model of alternative replication (Lederberg, 1955);

the "switch" theory (Chase and Doermann, 1958; Freese, 1957), combining the copy choice model and the concept of "effective pairing" (Pritchard, 1955), and recombination between side chains (De Serres, 1958).

In the light of recent evidence the gene has a number of mutable sites arranged in a linear order and separable by recombination ~~crossing-over~~. Alleles defective at different sites, heteroalleles (Roman, 1956), might be expected to differ in their mutation rates or physiological expression and it seems possible that there may be a number of alternative alterations at any particular site. The former expectation at least is realised for whilst some alleles of a biochemical mutant may make no growth on minimal medium, others make a little, and yet others will grow at one temperature but not at another. At the td locus of Neurospora (Yanofsky, 1956; Yanofsky and Bonner, 1956) allele specific suppressor genes have been identified and some alleles are distinguishable by their ability to use a certain precursor, or by their ability to produce a detectable derivative. Alleles at another locus (Giles, 1951, 1956) have been

shown to have different but characteristic rates of reversion.

Inter-allelic complementation, yet another criterion by which alleles can be shown to be differently defective, has been demonstrated in the heterozygote of yeast (Roman, 1956) and Aspergillus (Pritchard, 1955); in the heterocaryon of Neurospora (Fincham and Pateman, 1957; Giles, Partridge and Nelson, 1957) and by the abortive transduction in Salmonella typhimurium (Ozeki, 1956).

In the second part of Section A of this thesis inter-allelic complementation, in Neurospora, has been discussed with particular reference to the locus me-2, which was divisible into four sub-units. If these four sub-units could be translated into linear segments of the locus then it seemed probable that a correlation should exist between the map expressed by the pattern of complementation and that obtained by recombination between the mutated sites of the locus although recombination by unidirectional transfer may mask such a correlation. Suitable markers have been located on

either side of locus me-2 and inter-allelic crosses are fertile. The one disadvantage in the choice of this locus for studies on the fine structure of a gene, is that the identification of the enzyme cystathionase II (Fischer, 1957) depends on the chromatographic separation of the cystathionine cleavage product and cystathionine is expensive and not easily obtainable in a pure form. This section is, accordingly, restricted to the study of intra-genic recombination at locus me-2.

Theory of Methods

The analysis of recombination within a short region of the chromosome, in this case within a locus, is controlled by the use of outside markers situated close to, and on either side of, the region to be studied. To study recombination at a locus, m, crosses of the type $a\ m^1\ b^+ \times a^+\ m^2\ b$ are made where a and b are mutant biochemical markers, a^+ and b^+ their corresponding wild type alleles, and m^1 and m^2 independently occurring alleles at locus m. Recombinants prototrophic for the m locus are selected and their genotypes analysed with respect to the a and b markers.

If recombination occurs by crossing-over between the alleles $\underline{m}^1$ and $\underline{m}^2$ it is accompanied by recombination of the markers a and b in the absence of unexpected additional cross-overs between the m locus and the outside markers. An excess of recombinants of the genotype $\underline{a}\ \underline{m}^+\ \underline{b}$ would indicate the order $\underline{a}\ \underline{m}^2\ \underline{m}^1\ \underline{b}$ and conversely an excess of the recombinant genotype $\underline{a}^+\ \underline{m}^+\ \underline{b}^+$ would indicate the order $\underline{a}\ \underline{m}^1\ \underline{m}^2\ \underline{b}$.

Where recombination is the result of unidirectional transfer the distribution of the $\underline{m}^+$ recombinants amongst the four types of outside marker combinations ($\underline{a}\ \underline{b}$, $\underline{a}\ \underline{b}^+$, $\underline{a}^+\ \underline{b}$, $\underline{a}^+\ \underline{b}^+$) is approximately one of equality. Should recombination by this method be sufficiently frequent it is likely to obscure the results of intra-genic crossing-over.

Experimental Methods

The loci tryp-4 and pan-1 were chosen as outside markers and a representative section of alleles at the me-2 locus were crossed to a stock, Y2198, 5531, mutant at both marker loci. From these crosses fertile cultures of the genotypes $\underline{tryp}\ \underline{me}\ \underline{pan}^+\underline{A}$, $\underline{tryp}\ \underline{me}\ \underline{pan}^+\underline{a}$, $\underline{tryp}^+\ \underline{me}\ \underline{pan}\ \underline{A}$

and tryp^{+} me pan a, were selected. Crosses were then made of the type tryp me^{1} pan^{+} x tryp^{+} me^{2} pan where me^{1} and me^{2} are alleles at the me-2 locus. Similarly marked, self-crosses were made as controls.

All the crosses were made in two inch deep Petri dishes containing appropriately supplemented Westergaard's medium in which was embedded corrugated filter paper. The dishes were partially sealed by "sellotape", to prevent excessive drying of the medium, and incubated for approximately two weeks. At this stage spore shedding usually began and each Petri dish lid was replaced by a clean one, re-sealed, and the dishes then incubated for a further two weeks in an inverted position. In this way large numbers of ascospores, almost free of mycelial contamination, were deposited on the Petri dish lid. The spores were washed off the lid as a suspension in 0.1 per cent agar, for ascospores remain evenly distributed in this concentration of agar.

The number of spores per ml. of suspension was estimated by counting the number of spores in a 0.01 ml. drop of suspension on a microscope slide. A drop of this size could be conveniently enclosed by a cover slip

and lines were drawn on the under side of the slide to facilitate counting. From this count and a rough estimate of the spore viability the dilution necessary to give approximately 20,000 viable spores per ml. was calculated. The appropriately diluted spore suspension was treated for fifty minutes in an oven at 56°C.

Large numbers of spores from each cross were screened by use of a layer plating technique similar to that employed by Newmeyer (1954). A lower layer of approximately 15 ml. of 1 per cent sorbose medium, supplemented with tryptophan and pantothenic acid at the usual concentrations, was poured into Petri plates. These were kept usually for three days to allow the surface of the medium to dry before the upper layer was added. Aliquots (0.1 ml.) of heat treated spore suspension were transferred to tubes containing approximately 2 ml. of molten 0.5% agar medium and the contents of each tube were evenly distributed as the upper layer of a plate. All plating was carried out on a level sheet of glass.

To estimate the number of viable ascospores screened some plates were supplemented with methionine, tryptophan

and pantothenic acid. The upper layer of these plates contained 0.1 ml. of spore suspension diluted to $\frac{1}{10}$ of the previous concentration. The spores making growth on these plates were counted. Three or four such plates were counted for each experiment and the average number ($\bar{x}$) of viable spores per plate obtained. The number of spores in one plating experiment was estimated as $10 \times \bar{x} \times n$ where n is the number of plates screened.

The plates were incubated and after 40 hours the majority of the methionine independent colonies were first visible to the naked eye. A one per cent concentration of sorbose produced compact easily identifiable colonies and observation under the microscope confirmed that these colonies were growing from single ascospores. Each colony was transferred to a slope of Vogel's medium supplemented with tryptophan and pantothenic acid. The plates were re-examined after a further 8 hours incubation and any additional colonies were isolated.

Wherever heterocaryon complementation had been detected crosses involving these alleles yielded pseudo--wild types (PWT). In most crosses PWT's were not detectable until after three or four days incubation and

PWT cultures did not produce conidia within seven days of isolation on medium lacking methionine. However, PWT's from a cross between the alleles P169 and P99, which complement well in a heterocaryon, were first detectable after 48 hours of incubation and when isolated on medium lacking methionine they were not noticeably different from true wild types. All isolates from crosses involving these two alleles were tested to determine whether they were true or PWT with respect to the methionine locus.

Conidia, from each isolate, were spread on sorbose medium lacking methionine but appropriately supplemented with tryptophan and panthothenic acid. A number of conidia failing to grow on this medium were transferred to medium supplemented with methionine in addition to tryptophan and pantothenic acid. It was then determined which, if any, of these three supplements was necessary for the growth of each extracted culture. Where cultures of both me-2 alleles were recovered, it was concluded that the isolate was a PWT rather than a prototrophic recombinant. Conversely no methionine auxotrophs were

extracted from isolates of true wild type recombinants.

Results

The mutants used in this study and the complementation groups which they represent are listed in Table XVII. Group A contains the mutants which gave no complementation with any of the alleles studied. Groups B, C, D and E are defective in the four respective sub-units or segments into which the locus was divided, and group F is defective in the D and E segments.

Table XVII

Complementation group.	Mutants studied.
A	K5, P2
B	P169
C	P81
D	K44
E	H98, P99, P174
F	K23

(i) Self-crosses. Some of the self-crosses were not very fertile due mainly to the poor viability of the spores, but this is probably the result of associated viability factors rather than a property of the ~~locus~~ genes

themselves
~~itself~~. The number of viable spores screened for each self-cross is given in Table XVIII. In all the crosses only one prototroph was observed and since this carried the pan-1 marker it was probably not a contaminant, but it is not known whether the prototrophy was due to a change at the me-2 locus or to a mutation at a suppressor locus.

Table XVIII

Mutant	Number of	
	viable spores	prototrophs
K5	91,620	1
P2	88,980	0
P169	32,513	0
P81	95,526	0
K44	47,709	0
H98	73,800	0
P99	124,776	0
P174	50,630	0
K23	48,275	0

The results of the self-crosses indicate that the alleles studied, with the possible exception of K5, are

Table XIX

Analysis of prototroph frequencies from crosses between alleles of different complementation groups.

Cross P1 x P2	Groups represented	No. of viable spores	pan-2 prototrophs		No. of prototrophs in the four classes				χ^2 for deviation of recombinant classes from equality	Probability
			number	frequency	parental		cross-over			
					P1	P2	++	pan tryp		
P81 pan x P169 tryp	C x B	528,440	9	.0017	0	2	7	0	7.0	< .01
P169 pan x P81 tryp	B x C	311,520	7	.0023	2	2	0	3	3.0	.10 - .05
P169 pan x P99 tryp	B x E	80,248	6	.0075	2	0	4	0	4.0	< .05
P99 pan x P169 tryp	E x B	276,417	14	.0051	3	4	0	7	7.0	< .01
K44 pan x H98 tryp	D x E	202,886	91	.045	6	39	6	40	25.0	< .01
H98 pan x K23 tryp	E x F	244,306	103	.042	42	13	30	18	3.0	.10 - .05
K23 pan x H98 tryp	F x E	334,590	109	.033	17	38	17	37	7.4	< .01
K44 pan x K23 tryp	D x F	84,384	4	.0047	0	0	0	4	4.0	< .05
P81 pan x K44 tryp	C x D	181,200	84	.047	30	12	31	11	9.5	< .01
K44 pan x P81 tryp	D x C	133,630	78	.058	9	34	7	28	12.6	< .01

stable and that prototroph formation in inter-allelic crosses would be the result of some mechanism other than reversion. It would, however, have been desirable to obtain more significant results by screening considerably greater numbers of spores.

(ii) Crosses between mutants representing different complementation groups. The analyses of these crosses are shown in Table XIX. The prototrophic colonies observed in each cross were all isolated and tested.

(a) Intra-genic crossing-over. The significant excess of methionine prototrophs in one of the two cross-over classes, and a reciprocal relationship in reciprocal crosses, is evidence for a linear order of alleles. By calculation of χ^2s it is shown that for six of the ten crosses there is a significant deviation of the recombinant classes from a 1:1 ratio at the 0.01 probability level. From three (P81 tryp x P169 pan, P169 pan x P99 tryp and K44 pan x K23 tryp) of the four other crosses, insufficient numbers of prototrophs were isolated to obtain inequalities in the cross-over classes which were more significant than those listed in Table XIX.

However, in the reciprocal crosses of the first two of these, sufficiently large numbers of prototrophs were isolated in each case to give a significant excess of the expected cross-over type. In the remaining cross, H98 pan x K23 tryp, the excess of one of the cross-over classes is almost significant at the 0.05 probability whilst in the reciprocal cross the alternative cross-over class is significantly in excess at the 0.01 probability level.

From these data it is concluded that there is a linear order of the alleles and the sequence of the alleles and their outside markers can be determined. The order derived is tryp-4, D, E, B, C, pan-1 with the group F representative, K23, located close to K44 (group D). This is in agreement with one of the possible arrangements based on heterocaryon complementation, which requires groups D and E to be adjacent. Quantitative estimates of complementation from either growth or enzyme assays of heterocaryons have not been obtained and the only criteria by which the extent of complementation may be judged are the times taken for pseudo-wild type or heterocaryotic growths on minimal medium to become

visible and the extent of such growth in the absence of methionine. The order anticipated if heterocaryons between mutants in adjacent groups give a weaker growth than those between further separated mutants is B, C, D, E. The heterocaryon of mutants P169 and P99 (group B + group E) forms particularly quickly and both heterocaryon and corresponding pseudo-wild type make very good growth in the absence of methionine, but recombination data indicate that groups B and E are adjacent and the frequency of prototroph formation between mutants P169 and P99 is only 0.0056 per cent. Conversely, mutants P81 (group C) and K44 (group D) are the most widely separated according to the recombination data and yet PWT's extracted from crosses between these alleles make very slow growth in the absence of methionine.

(b) "Conversion". The cross H98 *pan* x K23 *tryp* was the first cross to be studied and a notable feature of the results was an unexpected, marked asymmetry in the distribution of the non-recombinant prototrophs between the two parental classes. Spores from this

Table XX

Analysis of non-recombinant prototrophs from crosses between alleles of different complementation groups.

Cross P1 x P2	No. of prototrophs in non-recombinant classes		χ^2 for deviation of P1:P2 from a 1:1 ratio	P
	P1	P2		
H98 *pan* x K23 *tryp*	42	13	15.3	.01
K23 *pan* x H98 *tryp*	17	38	8.02	.01
K44 *pan* x P81 *tryp*	9	34	14.5	.01
P81 *pan* x K44 *tryp*	30	12	7.7	.01
K44 *pan* x H98 *tryp*	6	39	24.2	.01
K44 *pan* x K23 *tryp*	0	0	-	-

cross were germinated on medium supplemented with methionine, pantothenic acid and tryptophan, and growing spores were isolated at random. The genotypes of the isolates were tested, but there was no excess of either the pan-1 or tryp-4 marker, thus indicating that differential viability of the markers was not responsible for the asymmetry.

In the cross P81 tryp x K44 pan a similar asymmetry was encountered suggesting that the inequality was not the result of chance. The results of the two reciprocal crosses (Table XX) confirmed the unequal distribution, but in each of these crosses the excess of prototrophs was in the alternate parental class, indicating that the effect was a characteristic of the me-2 alleles rather than the markers or any associated factors. Recombination by unidirectional transfer apparently occurred more frequently at some sites than others. The mutants H98 and P81 appeared more prone to conversion than mutants K23 and K44.

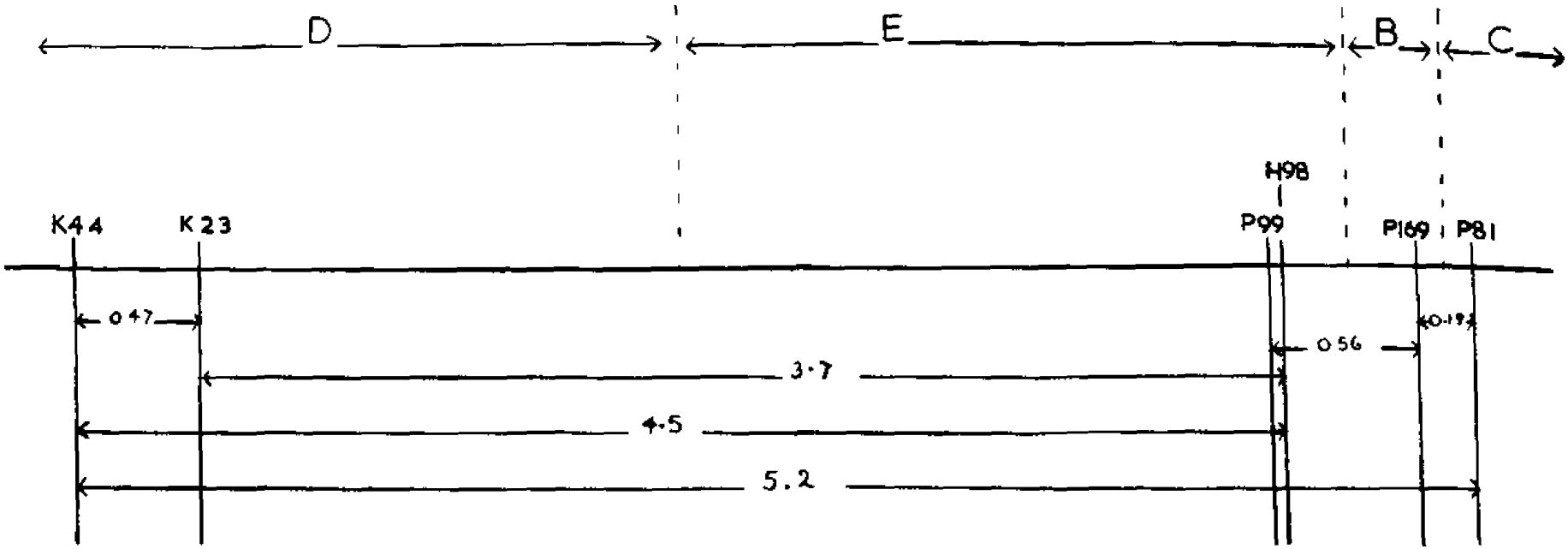
If mutants m^1 and m^2 have low conversion rates and a mutant m^3 a high conversion rate then in the crosses tryp m^1 pan$^+$ x tryp$^+$ m^3 pan and tryp m^2 pan$^+$ x

tryp$^+$ m^3 pan a marked asymmetry in the distribution of the non-recombinant prototrophs would be expected with the excess in the parental class carrying the pan marker. Recombination by conversion would be expected less frequently for the cross tryp m^1 pan$^+$ x tryp$^+$ m^2 pan, and asymmetry in the two parental classes, if present, would be less distinct.

At this time little information was available concerning the genetic map of locus me-2 and mutants K23, K44 and H98 were chosen as m^1, m^2 and m^3 respectively. The cross m^1 x m^3 has already been discussed, for it indicated a high conversion rate for the m^3 allele. The cross m^2 x m^3 showed, as anticipated, a much more frequent conversion at the m^3 site compared with the m^2 site. Cross m^1 x m^2 provided no relevant information, for few prototrophs were obtained.

In order that a test of the type outlined above may be performed it will be necessary to choose three mutants with sufficient recombination between any two of them to allow isolation of adequate numbers of prototrophs from each of the three crosses. This may

Fig VI Genetic map of locus *me*-2



Distances are expressed as the frequencies of methionine prototrophs per ten thousand viable spores plated.

be possible using the mutants H98 and P81 as alleles with high conversion rates and either K44 or K23 as the allele with a low conversion rate.

(c) Mapping within locus me-2. Using the data in Table XIX the genetic map of the locus me-2, shown in Fig. VI, has been constructed. The distances between alleles are expressed in terms of the frequency of methionine prototrophs per ten thousand viable spores. Where data are available for the reciprocal crosses involving two alleles, the frequency of prototrophs formation is calculated as the weighted mean. The data lack results from the cross P99 (group E) x K44 (group D) and it is, therefore, not possible to sum the constituent recombination frequencies P81(C) x P169(B) + P169(B) x P99(E) + P99(E) x K44(D) for comparison with the frequency obtained from the cross P81(C) x K44(D). A larger value is obtained for the latter cross than for crosses between any other alleles and it exceeds the sum of the available constituent crosses. Thus, it seems probable that the prototroph formation frequencies are additive and not markedly distorted by additional factors affecting recombination

Table XXI

Analysis of prototroph frequencies from crosses between alleles of the same complementation group.

Cross P1 x P2	Hetero-caryon group	No. of viable spores	me-2 prototrophs		No. of prototrophs in the four classes			
			Number	Frequency	non cross-over		cross-over	
					P1	P2	++	pan tryp
K5 pan x P2 tryp	A	179,080	59	.033	17	16	18	8
P2 pan x K5 tryp		146,220	62	.042	19	25	8	10
P174 pan x P99 tryp	E	448,367	7	.0016	2	3	1	1

frequency.

The boundaries between the segments of the locus are chosen arbitrarily as being mid-way between neighbouring representatives of adjacent segments. More precise information concerning the positions of such boundaries, if definite boundaries exist, requires the mapping of additional alleles.

(iii) Crosses between mutants within the same group. The results from crosses between alleles in group A and between alleles in group E are given in Table XXI. Methionine prototrophs were isolated from each of the three crosses.

Discussion

Consideration of the markers carried by the methionine prototrophs isolated from inter-allelic crosses at the me-2 locus indicate that recombination occurs by intra-genic recombination and by unidirectional transfer with approximately equal frequency. An excess of methionine prototrophs in one of the cross-over classes with the excess in the alternative cross-over class when the cross is reciprocally marked, is evidence

for a linear order of alleles. This property has been utilised in the determination of the sequence of the alleles studied.

The functional test of heterocaryon complementation divided the locus into four sub-units and evidence from the genetic test of crossing-over suggests that the complementation map can be correlated with the genetic map. Alleles in the same complementation group are localised in the same region of the genetic map and genetic data afford an arrangement of the four sub-units in a linear order which complies with one of the possible orders suggested by the complementation data.

A genetic map (Figure VI) of the me-2 locus has been constructed and, although only a small number of alleles can, as yet, be represented on this map the order from cross-over data is in agreement with the order derived from consideration of the prototroph frequencies. The present data are in agreement with the principle of additivity expected if recombination is the result of a genetic exchange. If the prototroph frequencies are in fact an estimate of the linear distance between alleles, the sub-units of the locus must vary in length, for in

locus me-2 group B must be small in comparison with group D and, or group E. The assumption that the genetic length of a segment may reflect the number of mutational sites appears quite reasonable and may account for the finding that of the twelve alleles capable of complementation two were located in groups B and C, and ten in groups D, E and the overlapping group F. That some complementation groups are represented by many alleles and others by only a few may be a general occurrence, and has been observed for example at the his-3 locus (Catcheside, 1959), the arg-1 locus (Catcheside and Overton, 1958) and locus ad-4 (Woodward et al., 1958) in Neurospora crassa. Differences in mutation rates, in the numbers of mutable sites, or in a combination of these two factors could be responsible for these distributions.

The occurrence of a high prototroph frequency in the cross between two group A alleles (K5 and P2) substantiates the view (Roman, 1956) that alleles in group A do not in general carry overlapping deficiencies. Furthermore, the prototroph frequency observed in this cross indicates that these two alleles are not confined

to a short region of the locus. This is in agreement with the finding of Case and Giles (1958) that the group A mutants are scattered throughout the length of the locus and some may even be homoallelic with the complementing alleles of groups B or C. Data from recombination and complementation tests at loci with more than two sub-units suggest that a mutation within a segment B may result in a mutant which falls within group A, group B, or a group E that overlaps segment B and one or more adjacent segments. An example of the latter type is furnished by mutant K23 (group F) located in segment D, but whose range overlaps segments D and E. These findings may be interpreted as evidence that both the precise location of a mutation and the nature of the mutational change determine the form and degree of alteration of the gene product's function.

Prototroph formation in a cross between two group E mutants confirms that these mutants have not got overlapping deficiencies in segment E. The recombination data generally substantiate the conception that the mutants, whether capable of complementation or not, are each defective at one of the many possible linearly

arranged sites within a chromosome region (i.e. the locus) which specifies a unitary function.

The hypothesis of Roman (1958) that the determinants of different genetic functions may overlap, so that mutation in the common segment can affect two different functions, is not tenable at a locus, such as me-2, for here a group A mutation would necessitate three simultaneous mutations, one in each of the three possible common segments.

Roman's hypothesis is even less applicable to the interpretation of the ad-4 locus of Neurospora crassa (Woodward, Partridge and Giles, 1958) which has been represented by a complementation map of seven sub-units. Mutants at this locus lack adenylosuccinase but the production of this enzyme in quantities of 3 - 25 per cent of that found in the wild type has been demonstrated with an approximate correlation between level of activity and distance of separation on the complementation map. Woodward (1959) reports a similar relationship from studies on enzyme complementation in vitro between adenylosuccinaseless mutants, and stresses the importance

of this correlation with the finding of a maximum enzyme activity of 25 per cent. He offers these data as evidence for a recombination process between gene products. Recombination between two templates could yield a maximum of 25 per cent of wild type templates. Further, the greater the distance between two defective sites, the greater is the probability of recombination.

The evidence available suggests that there is no correlation between map distance and restoration of enzyme activity at locus me-2. However, in the absence of enzyme and growth assay data a definite conclusion cannot be reached. From studies of the effect of am mutants of Neurospora crassa on the formation of glutamic dehydrogenase Fincham (1959) provided evidence for the presence of "hybrid" enzymes in heterocaryons comprising mutants capable of complementation. The formation of a "hybrid" enzyme, with properties different from those of wild type glutamic dehydrogenase, excluded a process of recombination at the gene product level yielding a wild type product or template capable of

catalysing the synthesis of an enzyme not qualitatively different from that of the wild type.

A further important aspect of prototroph formation in inter-allelic crosses is the mechanism of recombination by unidirectional transfer. The observation that some alleles at locus me-2 give rise to prototrophs by a non-conventional mechanism more frequently than others, favours a conversion hypothesis. However, the additivity of prototroph frequencies suggests that regardless of the mechanism of this recombination, the probability of its occurrence is a function of the distance between the two mutant sites involved. This additive behaviour is observed at the pan-2 locus in Neurospora crassa (Case and Giles, 1958) and at the ad-7 region in Schizosaccharomyces pombe (Leupold, 1958). Recombination of a non-reciprocal type probably occurs at both of these loci and these data, therefore, suggest that aberrant recombination involves a transfer of genetic material.

Additional evidence favouring a copy-choice hypothesis rather than directed mutation is given by Giles (1958) from studies at the ad-4 locus of Neurospora. Enzyme assays on cultures derived by reversion at the ad-4 locus

indicated that certain mutational changes may place permanent upper limits on the degree to which enzyme restoration can be achieved. Prototrophs assumed to be formed as the result of "conversion" exhibited enzyme activities equivalent to that of wild type, thus supporting the view that reverse mutations in conidia arise by a mechanism different from non-reciprocal prototroph formation at meiosis.

A surprising feature of the distribution of the prototrophs within the four classes was the relatively high number of prototrophs within the less frequent cross-over class, for if the outside markers are close to the locus, the number of prototrophs in this class should be negligible. Extension of these studies of random spores in conjunction with data from ordered asci may help in the elucidation of the mode of origin of both the unexpected reciprocal recombinants and the non-reciprocal recombinants. Confirmation should also be obtained that at least some of the excess cross-over class arise by intra-genic recombination.

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