Tests for Complete and Mosaic lethal Mutation in *Neurospora crassa*.

By

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INTRODUCTION.

Gene mutation may arise through changes in the already existing gene, or through an error in the assemblage of the daughter gene.

If the change in the existing gene affects the entire gene it would give rise to a wholly mutated clone of cells, as has been found to be the case for the great majority of mutations induced by radiation in Drosophila melanogaster in which the whloe body is involved. However, some of the induced mutants involving visible gene mutations were seen to be mosaic (Muller 1927, 1928a). These were shown to be due to changes in the already existing gene. To account for such cases of mosaicism, Patterson (1933) postulated the presence of a proportion of already split chromosomes at the time of treatment. Muller (1940) assumed that a single stranded chromosome at the time of treatment subsequently gave rise to a mosaic at the time of replication when of two broken chromatids only one underwent restitution. This theory, however, could not explain mosaics which were apparently due to gene mutation and not to chromosome breakage and reunion.

The demonstration of the double stranded nature of the genetic material (Watson and Crick 1953) has lead to the possibility of a mutation which affects one strand of a double stranded gene. A new pattern would thus be established, while the original gene still capable of forming replicas would remain, and so a mosaic would result. So mosaicism resulting from a change in the original gene could now be explained. However, the existence of a complete mutation now became more difficult to account for on this model since it was difficult to visualise how two strands could mutate at exactly corresponding points at such a high frequency.

A system was evolved from Atwood's method with the aim of determining the best conditions under which

mutations that result in a wholly mutated clone of cells and those that result in a mosaic could be distinguished operationally and comparatively studied.

The system most adaptable to the detection of mosaicism is one in which a mutation affecting morphological, colour or fermentation characteristics occur, since they become visible on the appropriate media. So the use of a selective procedure to eliminate the normal component of a mixed colony is avoided. This is the case on Levene's eosin methylene blue media where a lactose fermenting strain of E. coli gives dark red colonies, and non-fermenting mutants are detected by the presence of white sectors. Similarly, in Schizosaccharomyces pombe of genetic constitution ad_6 ad_7 , red pigment accumulates when it is grown on limiting adenine. Mutations at ad_1, ad_3, ad_4 and ad_5 loci will lead to the non-accumulation of red pigment, and so white sectors may be detected.

A replica plating technique has also been used for the detection of mosaicism. Kaudewitz et al (1958) detected the presence of prototrophic sectors in the auxotrophs which arose during P^{32} decay in E. coli. The number of replicates necessary to detect infrequent mutation and the possibility of missing very small mutant sectors are the disadvantages of this method.

The use of a selective procedure to obtain a mosaic recessive lethal in homozygous condition in Drosophila is used for the detection of mosaicism. In this case a treated male produces offspring some of which are heterozygous for a lethal mutation. They are allowed to mate with each other in order to obtain progeny homozygous for the mutation which will allow it to be detected. This is the same principle used in Atwood's method for Neurospora crassa where recessive lethals are scored in the methionineless nucleus of a balanced heterokaryon between arginine-less and methionineless, by allowing the formation of the homokaryon on methionine supplemented medium (Atwood, Muaki and Pittenger 1953).

The methionineless nucleus also carries the gene for amycelial which produces a strikingly abnormal growth habit. So a lethal in the methionineless nucleus can be detected by the absence of amycelial colonies. The advantage of this system is that it utilises a two component heterokaryon, thus it allows a mutation in one nucleus, which would normally be lethal, to be masked by the other when they exist as a heterokaryon. This lethal may be unmasked by the formation of the homokaryon, thus avoiding the complications of the sexual process necessary for the detection of a mutation in diploid organisms like Drosophila.

Possible sources of error in Atwood's method were first investigated, in order that they may be estimated where possible and taken into account. The presence of more than one methionineless nucleus in a heterokaryotic conidium results in deviations of the relative frequency of complete lethals and mosaic lethals found from their true values. Hence nuclear counts on different media were done with the aim of determining the medium on which the greatest proportion of conidia contain two nuclei. Then the frequency of nuclear types was determined, which together with the nuclear count data was used to correct for the effect of heterokaryotic conidia with more than one methionineless nucleus. As the operative nuclear count, and thus the corrections made, depend on whether or not the mutagen used acts by nuclear killing, attempts were made to estimate the amount of nuclear killing after the treatments used.

Tests for the best technique for the detection of mosaics were carried out, and an indication of the selection operating on mutant and non-mutant sectors of a mosaic obtained by means of reconstruction experiments. As amycelial is such a drastic morphological modification, it is possible that it may be affected readily by a mutagen and therefore produce a lethal mutation easily. Therefore, an alternative strain was investigated with the aim of distinguishing the best conditions under which its markers are easily recognised. This strain was a balanced

heterokaryon between adenineless and histidineless nicotinic acidless, and carries the gene for the morphological type ragged in the histidineless nicotinic acidless component.

Atwood's method was adapted for the purpose of making a comparison between the frequency of complete and mosaic lethals induced after treatment of conidia with X-rays, Ultraviolet light and chemical mutagens. This was done because it seems probable that whereas sources of high energy like X-rays may affect all replicas of a particular sub-unit, only a proportion will be affected by weaker energy sources like ultraviolet light, and also for chemical mutagens which have long been known to produce mosaics (Auerbach 1946, 1947, 1949, 1958; Auerbach and Robson 1946).

The two chemical mutagens, nitrous acid and 2-chloroethyl methane sulphonate, were chosen because they were known to be highly effective mutagens, had been shown to produce mosaics, and some work had been done on their mode of action.

Nitrous acid has been shown to be an effective inactivating and mutagenic agent for infectious RNA of Tobacco mosaic virus when treated in vitro (Schuster and Schramm 1958); for E. coli (Kaudewitz 1959) and in vitro treatment of Pneumococcal DNA has been shown to bring about modifications which are transformed into mutants when the treated DNA is introduced into another bacterium (Litman et Ephrussi-Taylor 1959). It has also been found to be effective in phage T_2 and T_4 (Vielmetter and Wieder 1959) and in Neurospora crassa (Auerbach unpublished).

Tessman in phage T_4 has demonstrated the presence of mottled plaques after treatment with nitrous acid, some of which contained 50% mutant and 50% non-mutant phage.

This mutagen has been shown to act by oxidative deamination of the primary amines in the bases of the DNA, changing cytosine to uracil, adenine to hypoxanthine and guanine to xanthine. In phage T_2 it has been shown that deamination of the

bases adenine and cytosine shows the same response to changes in pH as mutation frequency, whereas for guanine the deamination rate parallels inactivation (Vielmetter and Schuster 1959).

2-chloroethyl methane sulphonate (CB1506) kindly synthesised by Dr. J.L. Everett (1956) at the Chester Beatty Research Institute, was the other chemical mutagen investigated.

This chemical has been found to be an effective mutagen in Drosophila melanogaster (Fahmy and Fahmy 1955, 1956; Auerbach 1957; Auerbach and Sonbati 1957; Purdom 1957), and Neurospora (Auerbach 1960, Kølmark unpublished). It has also produced mosaics in Drosophila melanogaster (Auerbach unpublished). It has a slow action in producing its mutagenic effect, as was shown by a study of its effect on the back-mutation of an adenineless strain (No. 38701) of Neurospora crassa where it was only fully effective after thirteen hours at a molar concentration of 0.1 at 25°C (Kølmark in Westergaard). The bromo derivative was considerably faster in action being fully effective after two hours, while the iodo derivative was immediately effective (Kølmark unpublished).

It is an alkylating agent, and is characterised by its ability to transfer alkyl groups under physiological conditions (Ross 1958). Brookes and Lawley (1961) determined the rates and extent of alkylation of RNA and DNA by a variety of alkylating agents, which did not, however, include this mutagen. They found that alkylation occurs at the N-7 position of the guanine molecule, monofunctional agents yielding 7-alkylguanine, and difunctional agents in addition yield di(guanin-7-yl) derivatives. They suggested that a possible relationship may exist between the alkylation of DNA and the biological properties of the alkylating agent.

The investigation of this chemical forms the second part of this work.

MATERIALS AND METHODS.

STRAINS: arg./ meth., amy.

This strain, obtained from Dr. K.C. Atwood, consists of a balanced heterokaryon between arginineless (29997A) and methionineless, and carries the gene for amycelial in the methionineless component (4894-422A). On minimal medium, growth is in the form of a heterokaryon. Homokaryotic methionineless amycelial and arginineless colonies grow only on plates supplemented with methionine and arginine respectively, on which heterokaryotic colonies are also present. Plate 1 shows the morphology of the heterokaryon and of methionineless amycelial colonies on methionine supplemented media.

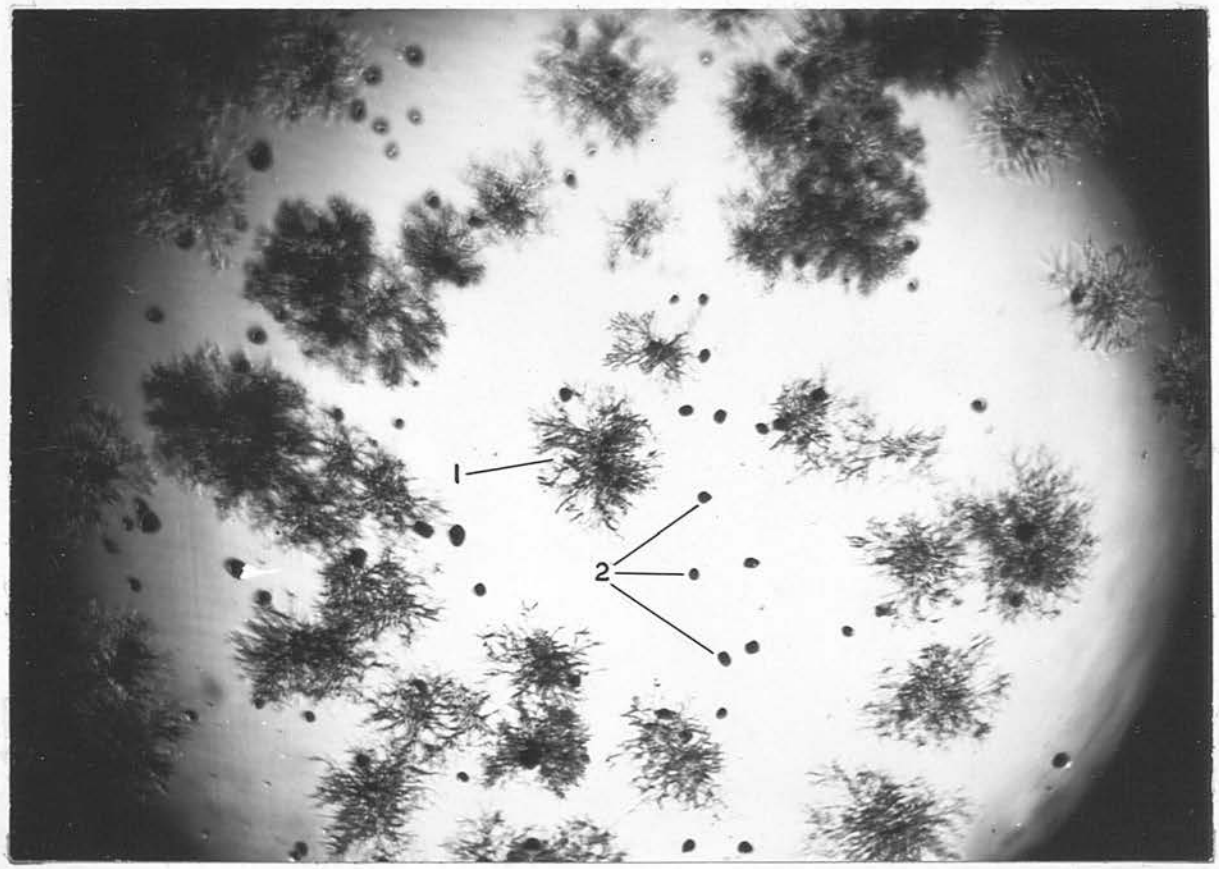
rg.hist.nic. / ad.

This strain, obtained from Dr. F. J. deSerres, consists of a balanced heterokaryon between adenineless , and histidineless nicotinic acidless, and carries the gene for the morphological type ragged in the histidineless nicotini acidless component. On minimal medium, growth is possible in the form of a heterokaryon. On histidine and nicotinic acid supplemented medium, the heterokaryon and the ragged morphology are shown. On medium supplemented with a limiting amount of adenine (0.5 mg/ litre), the heterokaryon and adenininеless colonies can be distinguished morphologically. When all three supplements are supplied, the heterokaryon, adenineless and ragged can be distinguished as in Plate 2.

MEDIA.

All stock cultures were maintained on slopes of minimal medium and stored at 25^{0}C.

Minimal medium contained 1.0 gms. sucrose, 1.0 ml. glycerol, 2 gms agar, 25 ml. Fries solution and 75 ml. distilled water.

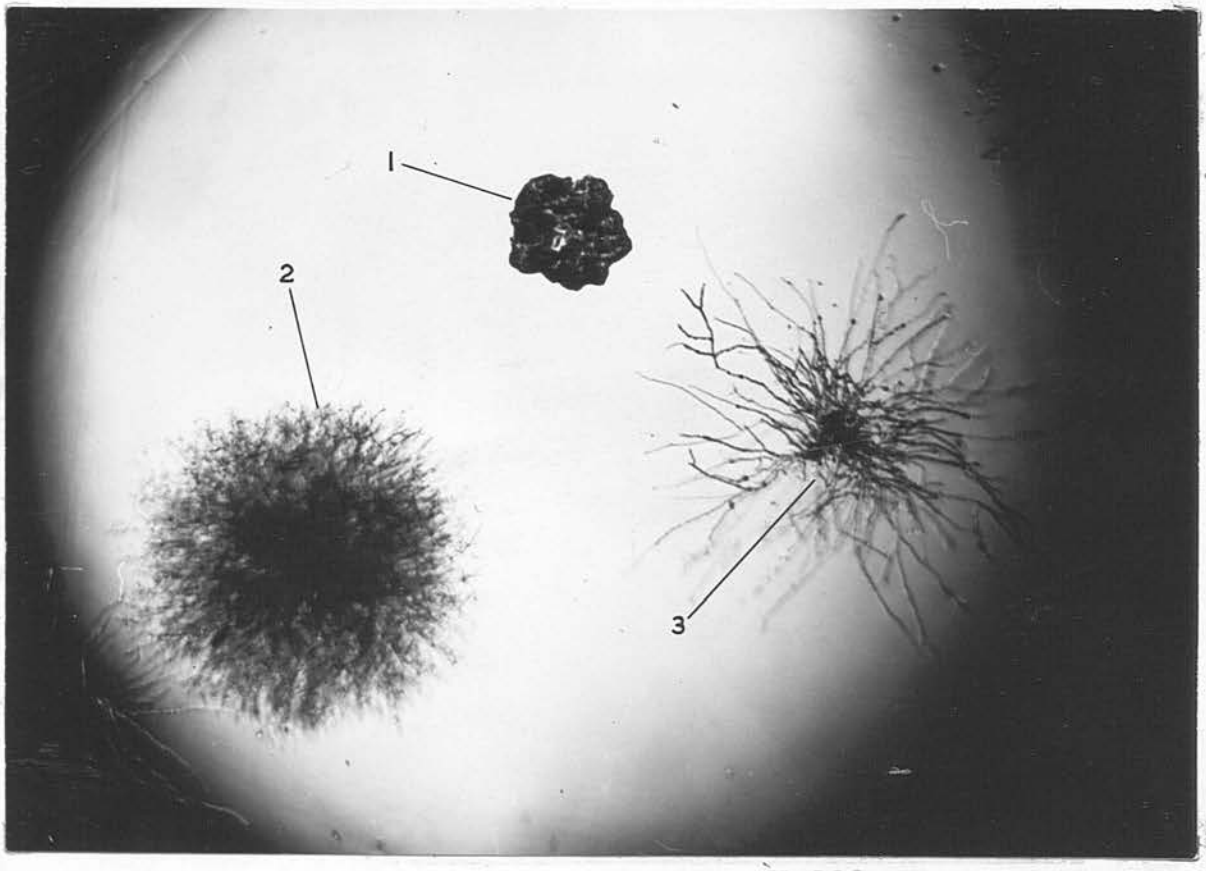


X 25

PLATE 1. Arginineless / Methionineless amycelial heterokaryon on minimal + methionine medium.

1 :- Heterokaryotic colony.

2 :- Methionine amycelial colony.



X 200.

PLATE 2. Ragged histidineless nicotinic acidless / adenineless heterokaryon on minimal + histidine + nicotinic acid + adenine (Limiting amount) medium.

1 :- Ragged histidineless nicotinic acidless colony.

2 :- Heterokaryotic colony.

3 :- Adenininеless colony.

The Fries solution used contained ammonium tartrate 100 gms., ammonium nitrate 20 gms., Potassium di-hydrogen phosphate 20 gms., magnesium sulphate 10 gms., sodium chloride 2 gms., Calcium chloride 2 gms., trace element solution 20 ml., biotin 80 µgms made up to 5 litres with distilled water.

The trace element solution used in the Fries solution contained 88 mg sodium borate, 268 mg cupric chloride, 970 mg ferric chloride, 72 mg manganese chloride, 37 mg ammonium molybdate, 4.168 mg zinc chloride, 8.806 mg zinc sulphate made up to 1 litre with distilled water. The Fries solution was the mineral requirement source in all media, and was used at a concentration of 25% .

Complete medium consisted of yeastral 0.25 gms., casein hydrolysate 0.1 gms., malt extract 0.5 gms, agar 1.5 gms., glycerol 1.5 ml per 100 ml media.

In some experiments, minimal medium was supplemented with adenine 40 mg., arginine 40 mg., inositol 20 mg, nicotinic acid 10 mg., histidine 100 mg., methionine 25 mg per litre.

The two types of plates used in Atwood's method were the 'Isolation' and'Squirt' plates. Both contained 0.1 gm. sucrose and 1.0 gm sorbose in order to secure a colonial growth habit of the conidia per 100 ml media. The 'Isolation' plates contained 2.0 gm agar, and the 'squirt' plates 2.5 gms agar per 100 ml in order that a firm medium would be secured for the 'squirting' techinique in which the plate is divided into sectors. Also, 'squirt' plates were supplemented with 25 mg/ litre methionine.

MUTAGENS.

X-rays

The source of X-rays was a Newton Victor GX10 Medium Therapy tube operated at 140 KV at a distance of 12.5 cm. for 1 hour. The tube was calibrated using a Baldwin-Farmer X-ray dose meter, and the dose rate was 40,00 r per hour. The conidial

suspension contained in a Petri dish with a 1 mm. thick aluminium lid, was agitated throughout treatment with a magnetic stirrer.

Ultraviolet light.

Ultraviolet light of wave length 2537 A^{o} from a Hanovia bactericidal lamp was used, at a distance of 42.8 cm. from the conidial suspension. The conidial suspension contained in a Petri dish was agitated by a shaker during irradiation.

Irradiation and subsequent handling of samples was done under low intensity yellow light to avoid photoreactivation.

Nitrous acid.

Effective treatment with nitrous acid was secured by using a pH of 4.4 at 25°C. This pH was obtained by a buffer consisting of 6.7 ml. 0.1M acetic acid mixed with 3.7 ml. 0.1M sodium acetate immediately before use. A control series of 2 ml. spore suspension and 4 ml. buffer, and a treated series of 2 ml. spore suspension, 2 ml. buffer and 2 ml. 0.05M sodium nitrite were used. The mixture was agitated during treatment, and treatment was stopped by dilution.

2-chloroethyl methane sulphonate. $Cl\ CH_2\ CH_2\ O\ SO_2\ CH_3$

Conidia were treated with 2-chloro ethyl methane sulphonate (CB 1506) for twelve hours at 25°C at a molar concentration of 0.1. This mutagen has a low solubility, and this solution is the highest molar concentration obtainable. These conditions were found to be effective in the treatment of an adenineless strain of Neurospora crassa for a study of the back mutation rate (Kølmark in Westergaard).

At the end of the treatment, the mutagen was

removed by washing twice with distilled water.

The conidial suspension used was at a suspension density of 1.5×10^6/ ml. for the first three mutagens. In the case of 2-chloroethyl methane sulphonate, in an experiment done in conjunction with Dr. Kølmark the suspension density was 3×10^6/ml. so that conditions in our separate treatments would be comparable. In all cases the syspension of conidia was prepared by filtration of the harvested conidia through cotton wool to remove fragments of mycelium, then washing was done twice to remove any traces of medium. Appropriate dilutions were made for plating on medium.

Experimental conditions which differ from the above will be indicated in the appropriate section.

ATWOOD'S METHOD.

This is a method which allows recessive lethals in Neurospora crassa to be scored (Atwood, Mukai and Pittenger 1953). The essence of the technique has already been mentioned on page 2 - 3, and is illustrated in Figure 1.

As shown, the arginineless /methionineless amycelial strain is first grown on minimal medium (I) on which it produces three kinds of conidia - homokaryotic arginineless conidia, homokaryotic methionineless amycelial conidia and heterokaryotic conidia (II). These conidia are harvested and after treatment plated on 'isolation' plates with minimal sorbose medium (III). Only heterokaryotic colonies can grow on the 'isolation' plates. The plating technique involves the use of tubes containing 2 ml. medium (1% agar) into which 1 ml. conidial suspension is pipetted; the contents are then emptied on top of previously poured 2% agar plates. Thus the colonies will grow discretely in a thin top layer, and can later be isolated.

These plates are incubated for 3 days at 30°C.

The sampling of large numbers of colonies from the 'isolation' plate is made possible by use of the 'punching' technique. For this technique punch tubes, clay based plates with minimal medium and perforated boards are used. The 'punch tubes' are 6 cm. long sections of glass tubing with a 3mm. inside diameter. They are dry sterilised with one end thrust in a covered tray of sand. The clay based plates are prepared by fitting filter paper in the bottom of a 14 cm. diameter Petri dish, then molding permoplast modeling clay to a uniform thickness over the paper. The covered Petri dish is autoclaved and cooled, and sterile medium poured on to the clay. The perforated boards for holding the 'punch tubes' in an upright position for incubation are dry sterilised and wrapped in tinfoil. Each board holds 120 tubes. 'Punching' is done by first coring the colony out of the isolation plate with the 'punch tube', and then pushing the tube into clay based plates with the minimal medium. The tubes are withdrawn and placed upright in the perforated boards, covered with tinfoil and incubated at 25°C to allow conidiation (IV).

The conidia are transferred to methionine supplemented sorbose medium by the 'squirting' method. This method saves both time in plating conidial suspensions, and plates since 5 isolates are 'squirted' per plate. For 'squirting' plate dividers and a Cornwall syringe are used. The plate dividers consist of open cylinders of polythene, 8 cm. high, just fitting the inside of a 9 cm. petri dish and having ten radial partitions. The divider is sterilised by dipping into boiling water for a few seconds. It is then pressed firmly into the agar plate. Three small protruberances on the bottom of the divider prevents the divider cutting the agar all the way through, thus facilitating its removal. By means of the Cornwall syringe, set to deliver 0.75 ml water, the contents of each 'punch tube' are 'squirted into

alternate sections of the divider. When all five alternate sections have been 'squirted', the plate and divider are inverted on a coarse screen and allowed to drain. The divider is then removed and the plate covered. Contamination from neighbouring sections is reduced by using alternate sections of the divider. A team of two, one using the dividers and the other the syringe, is best for the efficient performance of the technique.

The plates are scored for the presence or absence of amycelial colonies under a low power dissecting microscope after two days incubation at 30°C. A lethal in the methioninelessnucleus will be detected by the absence of methioninelessamycelial colonies (V).

MOSAIC LETHALS.

Only isolates in which the mutation affects all the existing methioninelessamycelial nuclei will give rise to immediately detectable mutants. Conidia with a lethal mutation induced in the methioninelessamycelial nucleus will not be detected if the lethal exists in a fraction of the genetic material of the methioninelessnucleus, or if a non-mutant methioninelessamycelial nucleus is present in the conidium. This is so because in both cases, the non-mutant amycelial units will give rise to viable amycelial colonies on methionine supplemented sorbose plates and will be indistinguishable from non-mutant isolates.

Isolates which are mosaic for a lethal may be detected by repeating the test on individual heterokaryotic colonies originating from a single treated conidium. The various techniques tried will be presented in the section on Experimental results.

DETERMINATION OF NUCLEAR NUMBER.

Nuclear counts on conidia were done using Huebschman's Azur A method with some modifications. The azur A-SO_2 complex specifically stains the aldehyde groups of the DNA released by acid hydrolysis in the same manner as the Feulgen reagent (Atwood

and Orinstein 1949, cited by DeLamater). The hydrolysis conditions are thus the critical factor influencing the staining result. Huebschman recommended hydrolysis in 1N hydrochloric acid at 55-60° for 9-14 minutes.

Thionyl chloride was found to be a more convenient source of sulphur dioxide than solid hydrogen sulphite recommended by Huebschman (1952).

The final staining procedure used was:-

1. Smear cover slip with glycerine albumen.
   (a) Dry conidia are patted on to the surface of the cover slip, using a platinum loop.
   (b) Wet conidia are placed on to the cover slip, spread evenly by rocking, then dried. Best results are obtained if they are now dehydrated slowly in alcohol.
2. Fix in Carnoy's fluid (3 parts absolute alcohol: 1 part glacial acetic acid) for 25 minutes.
3. Hydrolyse at 55°C ± 0.5°C for 12 ± 3 minutes, depending on age, in 1N hydrochloric acid, previously standardised. (Younger conidia are hydrolysed longer).
4. Stain in a mixture of 0.25% Azur A plus 2 drops thionyl chloride per 15 ml. If an excess of thionyl chloride is added a precipitate is formed. Stain for at least 1 hour.
5. Blot dry or dehydrate, then mount in Canada balsam.
6. Examine under oil immersion.

Throughout the staining procedure the cover slips are held in glass racks. This facilitates handling of the fragile cover slips, and minimises the possibility of washing off the conidia on the slip through rough handling.

RECONSTRUCTION EXPERIMENT.

In a number of experiments conidia were sampled from cultures grown from a mixture of lethal and non-lethal conidial suspensions, in order to estimate any possible selection against the lethal.

These conidia were then subjected to Atwood's test for recessive lethals.

RESULTS.

PART I. DETERMINATION AND CONTROL OF NUCLEAR NUMBER

A lethal in a methionineless nucleus can only be detected by Atwood's method if no other non-lethal methionineless nuclei are present in the conidium. Therefore the loss of scoreable lethals will increase directly with the proportion of heterokaryotic conidia which contain more than one methionineless nucleus. It is therefore important to determine the frequencies of conidia with various numbers of nuclei and the variation which exists in these frequencies as well as the factors which affect them.

(a) NUCLEAR COUNTS

Conidia were grown under conditions which might be expected to reduce nuclear number, and then examined cytologically to determine the nuclear number. These determinations were done with the aim of finding the conditions under which the highest proportion of heterokaryotic conidia would contain only one methionineless nucleus.

Huebschman (1952) found that whereas some dicarboxylic amino acids raised the average nuclear number, L lysine and glycine lowered it. Thus L lysine and glycine and the closely similar alanine were added to the medium to determine their effect on this strain in this laboratory.

Table 1 gives the cytologically determined numbers of conidia found to contain various numbers of nuclei under varying conditions of age and medium at 25°C.

There is no significant difference between nuclear counts done on conidia grown on minimal medium for 5, 6 or 8 days (χ^2 = 30.6 for 24 degrees of freedom, P is spproximately 20%). Similarly, there are no significant differences between 18 and 20 day old cultures grown on minimal medium. This is also the case for the two counts done on leucine and glycine respectively. However, the difference between counts done on 5-8 day old cultures on minimal, 18-20 day old cultures on minimal, 1% alanine, 0.9%

TABLE 1.

THE EFFECT OF AGE AND MEDIUM ON DISTRIBUTION OF CONIDIA CONTAINING VARIOUS NUMBERS OF NUCLEI

MEDIUM	AGE IN DAYS	NUMBER OF CONIDIA EXAMINED	NUMBER OF NUCLEI PER CONIDIUM							AVERAGE NUCLEAR NUMBER
			1	2	3	4	5	6	more than 6	
Minimal	5	300	111	115	50	12	6	5	1	2.02
	6	300	93	123	46	27	3	6	2	2.17
	6	300	81	134	55	16	9	5		2.18
	8	500	143	217	93	33	7	2	5	2.14
	18	300	65	126	88	15	5	1		2.24
	20	300	68	130	62	24	12	1	3	2.35
1% alanine	8	1000	111	405	295	114	46	11	18	2.62
0.9%leucine	8	500	109	239	112	29	10	1		2.19
	8	500	92	244	121	26	10	5	2	2.28
1% glycine	6	300	42	159	81	15	3			2.26
	8	300	36	148	94	16	6			2.36
complete	8	300	28	81	91	55	26	10	9	3.26

leucine, 1% glycine and complete medium is highly significant (P being < .01% in all cases).

From this Table it is seen that 5-8 day old cultures on minimal medium have the highest proportion of conidia with two nuclei. Thus conidia grown under these conditions were used in the experiments to be described and the % conidia with different numbers of nuclei are :-

NUMBER OF NUCLEI PER CONIDIUM.					
1	2	3	4	5	>5
31.14	42.07	17.43	6.29	1.78	1.29

(b) FREQUENCY OF NUCLEAR TYPES

The frequency of the two nuclear types will affect the relative frequency of homokaryotic methionineless and argininelessness, and of heterokaryotic conidia in the culture. It is possible to determine the frequencies of the various types of conidia by plating on minimal and supplemented media. Heterokaryotic colonies will be present on all plates, but homokaryotic methionineless and arginineless colonies will be present on plates supplemented with methionine and arginine respectively. By counting the colonies which appear when the same amount of conidial suspension is spread on minimal and on supplemented medium, the proportion of heterokaryotic and homokaryotic conidia in the sample can be determined. Since the methionineless amycelial colonies are morphologically distinct, they were scored separately where methionine was supplied. The results are given in Table 2.

It can be seen from Table 2 that the number of normal colonies on methionine medium is less than the number of colonies which grow on minimal medium, although the same number of normal colonies are expected on both. It was also noticed that on methionine medium the colony size varied considerably ranging to the limit of visibility, and it seems probable that methionine

TABLE 2

a) COLONY COUNTS ON DIFFERENT MEDIA (5 replicates each).

	MINIMAL	MINIMAL + METHIIONINE		MINIMAL + ARGININE	MINIMAL + METH-IONINE + ARGININE	
		Normal	Amycelial		Normal	Amycelial
Mean Number	149	117	75⁻	365	362	61

b) NUCLEAR TYPES

	HETEROKARYONS	HOMOKARYONS	
		METHIONINE AMYCELIAL	ARGININELESS
Mean number :-	149	95.5⁻⁻	216
% :-	32.35	20.74	46.91

- Before Correction factor described in text was applied
-- AAfter Correction factor described in text was applied

inhibits colony formation. This was also found to be the case by Atwood and Mukai (1955). Colony counts cannot determine whether the homokaryotic methionine amycelial colonies are similarly inhibited, but their similar variation in colony size leads one to assume that they are. This assumption was also made by Atwood and Mukai. Thus the amycelial count is adjusted by setting the count of normal colonies on methionine medium equal to that on minimal and making a similar adjustment for the amycelial colonies.

The adjusted plating data then reads:-

	Heterokaryon	Arginineless homokaryon	Methionineless homokaryon	Total
Number	149	216	95.5	460.5
%	32.36	46.90	20.74	100

These figures show a deficit in the % heterokaryon observed. This was also found by Atwood and Mukai for the same strain.

Prout et al have shown that there is a tendency for like nuclei to occur together as a result of incomplete nuclear mixing especially in the terminal branches of aerial hyphae, which is the site of conidial formation. This deviation from random distribution could account for the deficit of heterokaryotic conidia.

In view of this deficit of heterokaryotic conidia Atwood and Mukai suggested a method for the estimation of the proportion, p , of one nuclear type. This uses the equations:-

$$p = \frac{r\,(1-r) + a\,(\bar{n} - 2r)}{\bar{n}\,(1-r)} = 0.64$$

$$1-p = \frac{r\,(1-r) + b\,(\bar{n} - 2r)}{\bar{n}\,(1-r)} = 0.36$$

where a is the proportion of homokaryotic arginineless conidia, b is the proportion of homokaryotic methionineless conidia, r is the proportion of heterokaryotic conidia and $\bar{n}$ is the average number of nuclei per conidium. However, Klein (1958) found that the distribution of nuclei was random in heterokaryons constituted from pantothenicless, nicotinicless - 2 albino - 1, lysineless -3

and p-aminobenzoicless homokaryons. However, whether p is calculated assuming random distribution or by Atwood's formulae given previously, similar values are obtained (Atwood and Mukai).

This particular form of nonrandom distribution should not interfere with the relative proportions of heterokaryotic conidia which contain various numbers of methionineless nuclei. These proportions are shown in Table 3. From these proportions, and the various proportions of conidia which contain various numbers of nuclei it is possible to calculate the overall proportions of heterokaryotic conidia which contain 1, 2, 3etc methionineless nuclei. These are shown in Table 4.

Since complete lethals will only be detected in conidia with one methionineless nucleus, the proportion of complete lethals found, L , has been produced by lethal mutation in heterokaryotic conidia containing one methionineless nucleus, A_1. Thus the probability, P , of a lethal arising in a methionineless nucleus $= L / A_1$.

From P, one may calculate the amount of mosaics which are due to complete lethal mutations in conidia containing more than one methionineless nucleus. In conidia containing two methionineless nuclei (A_2), there is a probability, P , of a lethal being induced in each nucleus. This gives a total probability 2P of a lethal being induced in the conidium. However, P^2 proportion of the time a lethal will have been introduced in both nuclei and since Atwood's method will only indicate the presence or absence of mutation, $P^2 / 2$ mutations will not be separately detected. Thus the proportion of mosaics which will be formed by complete lethal mutation in conidia with two methionineless nuclei $= A_2 (2P - P^2/2)$. Similar calculations may be done for conidia containing 3, 4 etc methionineless nuclei. Mosaics produced in this manner may now be subtracted from the mosaics found; this leaves the mosaics produced by mosaic mutation (Table 7).

These true mosaics represent mosaic mutations which occur in conidia with any number of methionineless nuclei.

TABLE 3

FREQUENCY DISTRIBUTION OF CONIDIA WITH VARIOUS NUMBERS OF METHIONINELESS NUCLEI

NUMBER OF NUCLEI	NUMBER OF METHIONINELESS NUCLEI					TOTAL	PROPORTION WITH VARIOUS NUMBERS OF NUCLEI
	1	2	3	4	5		
2	.46					.46	.42
3	.44	.24				.68	.17
4	.38	.32	.12			.82	.06
5	.31	.34	.19	.05		.89	.02
6	.24	.33	.24	.10	.02	.93	.01

-TABLE 4-

% OF HETEROKARYOTIC CONIDIA CONTAINING VARIOUS NUMBERS OF METHIONINELESS NUCLEI

Number of Methionineless Nuclei:-	A_1 1	A_2 2	A_3 3	A_4 4	A_5 5
%	83.07	14.20	2.34	0.35	0.04

The probability, m , of a mosaic mutation occuring in a methionineless nucleus, the proportions of heterokaryotic conidia containing 1, 2, 3 etc methionineless nuclei(A_1, A_2, A_3 etc) , and the proportion of true mosaics found, M_m , are related by the following equation :-

$$\text{True Mosaics} = A_1 m + A_2\left(2m - \frac{m^2}{2}\right) + A_3 \frac{m(5m^2 - 9m + 18)}{6} + A_4 \frac{m(-13m^3 + 4m^2 - 36m + 48)}{12} = M_m$$

PART II INDUCED MUTATION

(a) Nitrous Acid.

1. DETERMINATION OF THE TYPE OF KILLING.

The type of killing is important from the standpoint of the corrections made for complete and mosaic lethals. If nuclear killing occurs, there would be a decrease in the fraction of conidia which contained more than one methionineless nucleus and the corrections applied would be overcorrections. Also, it is important to determine whether conidia with different numbers of nuclei were preferentially killed. If killing favoured the survival of conidia with greater numbers of nuclei, the fraction of conidia with more than one methionineless nucleus would increase, and the determination of the conditions under which the greatest number of heterokaryotic conidia contain 1 methionineless nucleus would be of no consequence.

Killing by X-rays has been shown to be nuclear in this strain (Atwood 1954). The killing due to ultraviolet light has been shown to be due to nuclear killing at low doses of irradiation and at higher doses, cytoplasmic inactivation is added to the process of nuclear inactivation (Goodman 1958). In view of this, the determination of the type of killing was done only for the mutagen nitrous acid.

If the killing of conidia takes place through the inactivation of nuclei, then at some stage before a heterokaryotic conidium is killed, it will become monokaryotic and as a consequence be a homokaryon. So with decreasing survival, there would be a decrease in the proportion of heterokaryotic conidia and an increase in the proportion of homokaryotic conidia. Therefore, the frequency of colonies on minimal should decrease more quickly than that on supplemented medium. This was tested by treating a conidial suspension with nitrous acid as described on page 10, removing samples at appropriate intervals, and immediatly diluting for subsequent plating at different survival levels. By plating treated conidia on minimal medium, methionine

supplemented medium and arginine supplemented medium the relative proportion of each conidial type can be determined at the different survival levels.

Concurrently with this experiment, the frequency of recessive lethals was determined at four different survival levels in order to check any possible relationship to nuclear killing. The results are given in Table 5 and Graph 1.

Table 5 shows that with decreasing survival there is no decrease in the proportion of heterokaryotic conidia found. A correction factor to compensate for the influence of methionine in the medium was made in the case of the methionine-less amycelial colonies as described in Part I. This table also shows that the % recessive lethals increased with decreasing survival.

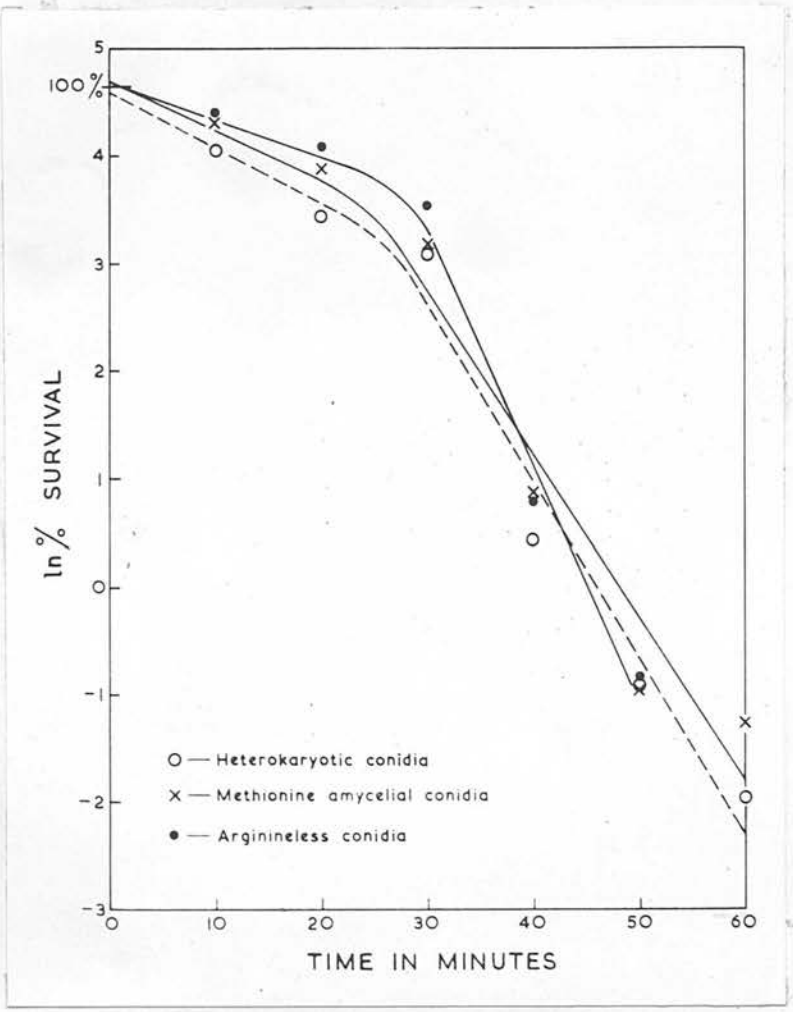
Graph 1 is a plot of $\log_e$ percentage survival against duration of treatment for the three conidial types.

The shape of these curves is similar to that obtained by Goodman (1958) for a micro-conidial strain of Neurospora crassa treated with ultraviolet irradiation. His interpretation was that at low doses conidia are killed by inactivation of nuclei, and at higher doses cytoplasmic inactivation is added to the process of nuclear inactivation. It is possible that a similar interpretation may be applied to Graph 1. The plating data which apparently gives no indication of nuclear killing would then have to be explained on the basis of a hypothesis put forward by Atwood (1954). He found that after treating conidia of the same strain used here with ultraviolet light, the estimate of the proportion of heterokaryotic conidia obtained from counts on minimal media differed from that obtained by isolating colonies from supplemented media and testing them for their ability to grow on minimal media. There was only a slight decrease in the proportion of heterokaryotic conidia found using the first method, while a rapid decrease, indicating nuclear killing, was obtained by the second method. He explained this on the basis of a 'recovery' hypothesis which suggested that on minimal medium, one nuclear type

TABLE 5.

THE EFFECT OF NITROUS ACID ON (a) SURVIVAL ON DIFFERENT PLATING MEDIA.(b) PROPORTIONS OF THREE CONIDIAL TYPES (c) RECESSIVE LETHALS.

	a			b			c
TIME OF TREATMENT IN MINUTES	% SURVIVAL ON PLATING MEDIA OF HETEROKARYON	METHIONINELESS AMYCELAIL	ARGININELESS	% CONIDIAL TYPES HETEROKARYON	METHIONINELESS AMYCELIAL	ARGININELESS	% LETHALS
0	100	100	100	43.19	24.54	32.27	0
10	58.3	75.8	81.5	35.94	26.56	37.50	0.77
20	30.5	48.6	58.9	29.88	27.00	43.12	
30	21.8	24.3	34.4	35.57	22.54	41.89	
40	1.5	2.37	2.21	32.61	30.30	37.10	5.5
50	.40	.39	.41	38.50	21.30	40.20	
60	.14	.28					10.5



GRAPH 1. The effect of nitrous acid on the survival of the three types of conidia.

has to await the recovery of the other nuclear type before growth is possible. However, on supplemented medium, growth is immediately possible in the form of a homokaryon. Therefore, by plating first on supplemented medium, the deficit of heterokaryotic conidia can be shown. However, there is a constancy in the relative proportions of heterokaryotic and homokaryotic conidia that would make it very difficult to apply a similar 'recovery' interpretation to the results of this experiment unless recovery is unrelated to dose or survival.

For that matter, even if the 'recovery' hypothesis were applicable, nuclear killing would only become a reality when plating on supplemented medium which is not the case here.

2. DETECTION OF COMPLETE AND MOSAIC LETHALS.

Complete lethals were detected by Atwood's method as described on page 11. Mosaic lethals which contain mutant and non-mutant methionineless amycelial nuclei, are not detectable by this method as the viable methionineless amycelial nuclei give rise to colonies with the amycelial morphology. If mosaics are to be detected the mutant and non-mutant nuclei must be given the opportunity to segregate so that only mutant nuclei occur in a culture, thus making it possible for the lethal to be detected. Therefore, any method for the detection of these mosaic lethals utilises the technique of multiple sampling with the hope of achieving this segregation of the lethal from the non-lethal nuclei.

With this aim in view, macroconidia of Atwood's strain were treated with nitrous acid and various methods were tried in an attempt to detect mosaic lethals.

METHOD 1:- Eight sectors were sampled from the edge of a heterokaryotic colony growing on the 'isolation' plate. Sampling was done by means of a platinum spade, and the samples were placed in 'punch tubes' containing minimal glycerol medium. After conidiation, the samples were submitted to Atwood's test for recessive lethals and the eight sectors were scored

separately for lethals. This method is illustrated in Figure 1, Section C. For simplicity, the illustration shows only two sectors taken from a colony on the isolation plate. It was applied to 27 colonies, and none were found to be mosaics. This, however, may have been due to the growth habit of the colony making it unlikely that discrete lethal and non-lethal portions would develop and be sampled separately. A different method was therefore tried.

METHOD 2:- Discrete heterokaryotic isolates were sampled from methionine sorbose plates on which amycelial colonies also grew. This ensures that only non-lethals are submitted to the test for mosaicism, and thus has the advantage over Method 1. Two techniques were used for picking isolates. (a) Heterokaryotic colonies were sampled by picking colonies with a platinum spade under the microscope, and transfering to 'punch tubes'.
(b) Heterokaryotic colonies were cored out using a 'punch tube' with the aid of a magnifying glass. This increases the speed of sampling. For this method a sparse growth of colonies is necessary in order to obtain discrete isolates. Therefore the conidia from one punch tube were squirted on a single 're-isolation' plate and then the plate was washed with 9 ml. water, in portions of 3 ml. each to remove excess conidia. For poorly conidiating cultures less water was used for washing. The 'punching' was done three days later when all the viable conidia have germinated.

After conidiation, the 're-isolates' in the 'punch tubes' were submitted to Atwood's test for recessive lethals.

This method is illustrated in Figure 1, Section B.

Mosaics were detected by this method. Using Method 2 a, and sampling ten isolates from each non-lethal colony, 9 colonies out of a total of 48 tested were found to be mosaic lethals. Using Method 2 b and sampling twenty isolates from each non-lethal colony on a methionine supplemented plate, 50

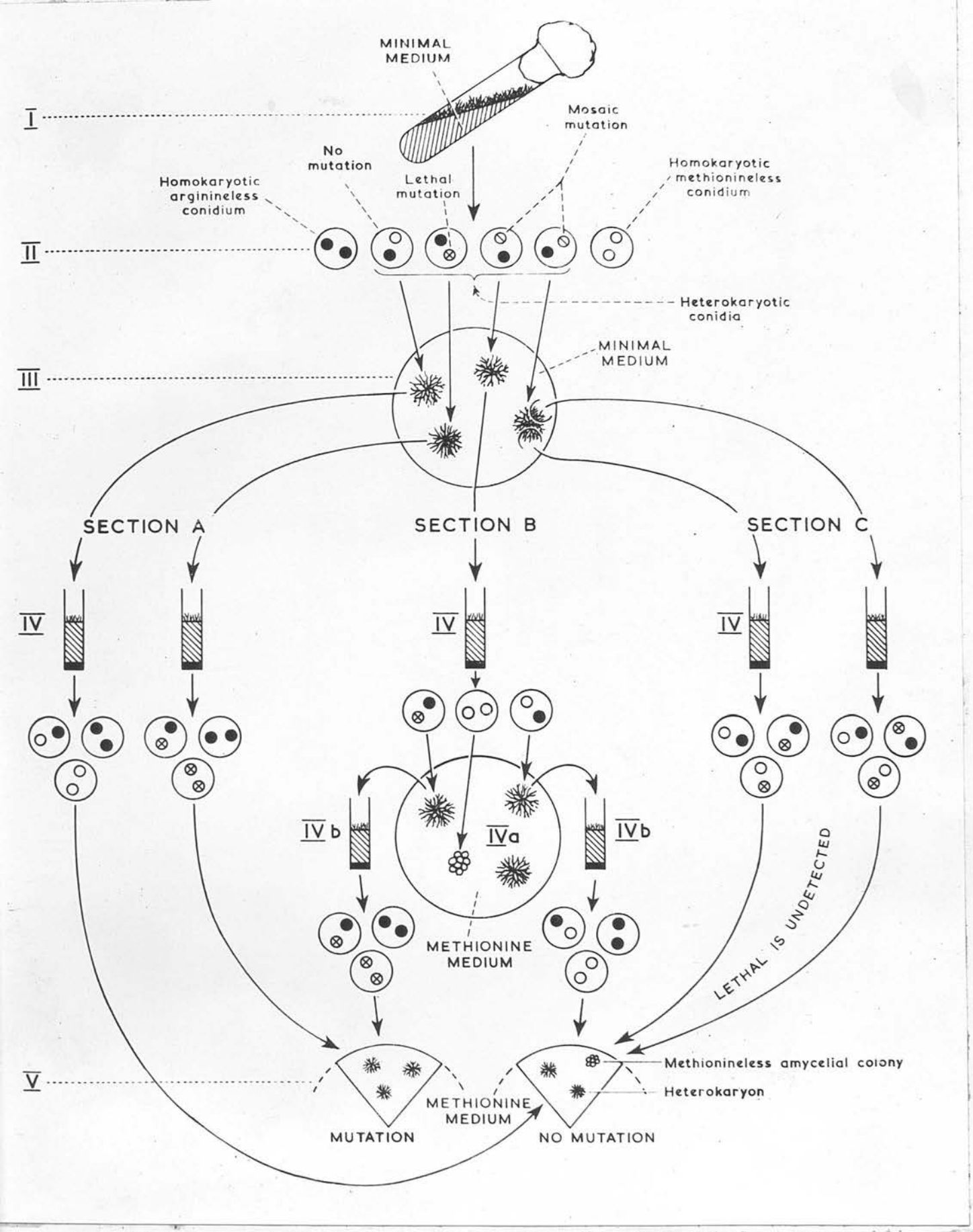


FIGURE 1:- I. Slant with macroconidia. II. Harvested macroconidia III. 'Isolation'plate. IV. 'Punch' tubes. IVa. Second 'isolation' plate IVb Second 'punch' tube. V. 'Squirt ' Plate. Section A:- Detection of a lethal. Section B:- Detection of Mosaic Method 2. Section C:- Detection of Mosaic Method 1.

colonies out of a total of 75 were found to be mosaic lethals.

The results of an experiment using the sampling method described under Method 2 b are given in Table 6, Experiment I. This table shows the frequency of complete and mosaic lethals observed, and also after the correction due to conidia containing more than one methionineless nucleus was made. The method of doing this correction has been described in Part 1, and details of mosaics formed as a result of 2, 3, 4 methionineless nuclei being present in a conidium are given in Table 7.

These results show that 9.3% lethals and 69.8% mosaic lethals were observed among survivors after treatment with nitrous acid for 30 minutes. After correction was made for conidia containing more than one methionineless nuclei, the frequency of mosaic lethals was 65.8% Thus 4% of the mosaic lethals were due to complete lethal mutations in conidia with more than one methionineless nucleus. If this figure is added to the 9.3% lethals observed it will give the proportion of conidia in which complete lethal mutation is induced for a particular nuclear distribution. Thus a change in nuclear distribution would hinder comparisons and it was decided to calculate mutation per nucleus instead. These values are given in Table 7.

3. COMPARISON OF THE FREQUENCIES OF COMPLETE AND MOSAIC LETHALS AFTER TREATMENT WITH X-RAYS, ULTRAVIOLET LIGHT AND NITROUS ACID.

X-rays, which as a source of high energy are presumed to act on all the DNA strands, are expected to give few or no mosaics. Thus the frequency of mosaics in X-ray experiments with a high survival may be used as an independent check on the proportion of mosaics that are due to the presence of more than one methionineless nucleus in a conidium. Ultraviolet

TABLE 6.

THE EFFECT OF VARIOUS MUTAGENS ON THE FREQUENCIES OF COMPLETE LETHALS AND MOSAICS.

EXP.	SERIES	MUTAGEN	CONIDIA PUNCHED	LETHALS	% LETHALS	NUMBER TESTED FOR MOSAICS	MOSAICS	MOSAICS AS % OF TOTAL OBS.	MOSAICS AS % OF TOTAL COR.	% SURVIVAL
I	Control	Nitrous acid	340	0	0					
	Treated		516	48	9.3	75	50	69.8	65.78	0.5
II	Control		300	0	0					
	Treated	Nitrous acid	412	34	8.1	94	27	26.4	22.92	0.58
		Ultra-violet	323	122	37.8	80	33	25.7	11.09	2.83
		X-rays	227	77	34	69	14	13.4	0.07	11.08

DISTRIBUTION OF CONIDIUM CONTAINING VARIOUS NUMBERS OF NUCLEI (EXPERIMENT II)

NUMBER OF NUCLEI								TOTAL CONIDIA
1	2	3	4	5	6	7	8	
28	51	17	1	1	1	0	1	100

TABLE 7.

EQUATIONS USED IN CALCULATIONS	OBSERVED AND CORRECTED RESULTS			
	Exp I	Experiment II		
	% NO_2	%NO_2	%UV	%X-rays
FRACTION OF DETECTABLE LETHALS (L)				
$L = A_1P$	9.3	8.1	37.8	34.0
PROBABILITY OF LETHALS IN WHOLE GENOME (P)				
$P = \frac{L}{A_1}$	11.20	9.75	45.5	40.93
MOSAICS FORMED BY (1) Complete lethals in A_2 conidia = $(2P - \frac{P^2}{2})A_2$	3.09	2.70	11.45	10.44
(2) " " " A_3 " $= \frac{P\,(5P^2 - 9P + 18)}{6} A_3$	.74	.65	2.65	2.43
(3) " " "A_4 " $= \frac{P\,(-13P^3 + 40P^2 - 36P + 48)}{12} A_4$	.15	.13	.51	.47
(1) + (2) + (3)=M_L	3.98	3.48	14.61	13.33
MOSAICS OBSERVED (M)	69.76	26.40	25.70	13.40
MOSAICS FROM MOSAIC MUTATION (M_M) = M - M_L	65.78	22.92	11.09	0.07
PROBABILITY OF MOSAIC MUTATION IN WHOLE GENOME (m) is secured from solving				
$A_1m + A_2(2m - \frac{m^2}{2}) + \frac{A_3m\,(5m^2 - 9m + 18)}{6} + \frac{A_4m\,(-13m^3 + 40m^2 - 36m + 48}{12} = M_M$	57.5	19.5	9.39	0

light and nitrous acid being weaker sources of energy might possibly affect only a proportion of the replicas of a particular sub-unit and give a significant proportion of mosaic lethals.

These three mutagens were used to treat the same conidial suspension by the methods described on pages 9 -11. A conidial count to determine the frequency distribution of conidia containing various numbers of nuclei was also done.

The results are given in Tables 6 and 7, Experiment II. Sampling for mosaic lethals was done as in Experiment 1, the results of which are also included in these Tables.

From Table 6, a comparison of the observed mosaic lethals after nitrous acid treatment in Experiments I andII shows that the results differ widely, the figures being 69.8 and 26.4% respectively. One possible explanation is that the distribution of conidia with various numbers of nuclei might have been different in the two experiments. The nuclear count done in Experiment II showed that among conidia containing more than one nucleus the majority had two nuclei. However, nuclear counts were not done in Experiment I and the nuclear number may have been higher. Another possible explanation lies in the method of multiple sampling used. Samples were taken from colonies growing on methionine supplemented medium, where homokaryotic methionineless amycelial colonies as well as heterokaryotic colonies grow. It is possible that these two types of colonies may form a heterokaryon if they lie in close proximity. Heterokaryon formation between colonies, one of which carries a lethal and the other a non-lethal methionineless nucleus, would result in a heterokaryotic colony with an undetectable lethal. Thus there would be a reduction in the number of mosaic lethals detected. It is possible that sampling in the second experiment was done from plates which were more densely plated than in Experiment 1, and which would favour heterokaryon formation between lethal and non-lethal colonies. This may account for the different proportions of mosaic lethals found.

A direct comparison of the effect of the three mutagens used in Experiment II may be misleading since the survival levels are so different. However, the relative frequencies within a mutagen of complete lethals, mosaic lethals and non-lethals may be compared between mutagens.

In Table 7 the figures for complete and mosaic lethals are shown. The mosaic lethals are corrected for the error caused by conidia containing more than one methioninelessnucleus. The results are then expressed as the probability of mutation occuring in a methioninelessnucleus for both complete and mosaic lethals. In this form the results are comparable regardless of the nuclear distribution of the conidial sample used. Since the probability of mosaic mutation expressed as a percentage of the probability of all mutation is comparable between mutagens, the results are expressed in this form. These values are 83.7%, 66.6%, 17.1% and 0% for Experiment I nitrous acid, Experiment II nitrous acid, ultraviolet light and X-rays respectively.

This is the trend expected from the considerations on page 31. However, as explained in Part 1, the corrections applied would be overcorrections for a mutagen which acted by nuclear killing. Therefore for X-rays the figure given is lower than the true value, whereas those for ultraviolet light and nitrous acid will be nearer the true values.

4. SECTOR SIZE

If one assumes the double stranded nature of the genetic material to be correct, then the two main types of mutation mentioned on page 1 will give predictable results. In the first case where gene mutation arises through a change in the already existing gene, the mosaic produced will consist of a half mutant and a half non-mutant portion. In the second case where mutation arises through an error in the assemblage of the daughter gene, replication will produce four strands, one of which will be mutant thus leading to a mutant sector of one quarter.

These two expectations assume that the mutagen has an immediate action. If, however, there is a delay in the action of the mutagen it will have the effect of reducing the size of the mutant sector in both cases. However the difference in the original proportions on which these reductions will operate, may make it possible to decide between the two mechanisms from data on the relative frequencies of mosaics with various mutant sector sizes.

The results for the size of mutant sectors in the mosaics detected in Experiments I and II are given in Table 8. This shows that the proportion of lethals in 70-80% of the mosaics produced by all mutagens was in the 0-20% size class.

However, before speculating on the merits of the two mechanisms mentioned above, the possibility of selection against the lethal being the factor responsible for the low mutant sectors detected in the mosaics was tested.

5. RECONSTRUCTION EXPERIMENT.

The possibility that selection operating against the lethal sectors of a mosaic was responsible for the small size of mutant sectors detected in the mosaics, was tested by means of a reconstruction experiment. In this experiment heterokaryons were formed from equal proportions of conidia from lethal and non-lethal cultures. They were then submitted to Atwood's test to determine the relative proportions of lethal and non-lethal methionineless nuclei.

The lethals to be used were first tested to ensure that complete lethals and not mosaic lethals were used in the test. This was done by growing the lethals on complete medium where conditions favour conidia with the maximum number of nuclei. Under these conditions there is the greatest chance that conidia will form new combinations of nuclei with two or more lethals present, and viable homokaryotic amycelial conidia will be formed

TABLE 8

NUMBER OF MOSAIS CONTAINING A GIVEN % OF LETHALS AMONG 20 ISOLATES TESTED FROM EACH

% LETHALS	NITROUS ACID Experiment I	NITROUS ACID Experiment II	ULTRAVIOLET Experiment II	X-RAYS Experiment II
0-10	18	17	18	10
-20	20	3	5	1
-30	4	3	5	2
-40	4	0	0	0
-50	1	1	2	0
-60	1	2	1	1
-70	0	0	0	
-80	0	1	1	
-90	1		1	
-100	1			

as a result of complementation (Atwood 1954). Such conidia were not detected, so it was consluded that the lethals used contained only one lethal mutation.

Conidial suspensions of eight different lethals and one non-lethal were made and adjusted to the same suspension density. Then 2 ml. of each lethal were mixed separately with 2 ml. non-lethal suspension, and the mixture centrifuged and the supernatant decanted. A portion of the pellet was then transfered to sorbose agar plates. The plates were incubated to allow the conidia to coalesce, and 12 hours later an inoculum from the pellet was transfered to glycerol agar slants and allowed to conidiate. This is a modification of the method used by Pittenger et al 1955. The conidia were then submitted to Atwood's test for recessive lethals.

The results are given in Table 19, and show that in 6 out of 8 cases the % lethals recovered were less than 7.5%. In two cases no lethals were recovered. The incubation of the pellet of mixed conidia for twelve hours should give an opportunity for the hyphae of germinating conidia to coalesce and so form a heterokaryon between lethal and non-lethal nuclei. In the 2 cases in which no lethals were detected, it is probable that the inoculum did not contain a portion of any heterokaryon that was formed. However, in those cases where lethals were recovered, it is assumed that heterokaryon formation took place and the low proportion of lethals recovered was due to selection operating against the lethal fraction of the reconstructed mosaic.

Auerbach (1959) using the same strain has shown that during the initial stage of growth in a growth tube, 80% or more of the lethals accumulated over a period of 23 weeks at 30°C were lost. Therefore, it appears that during the initial stages of growth the division of mutant and non-mutant nuclei are not synchronous and conditions operate against the growth of nuclei with a lethal mutation. From this it appears that any improvements in the method used must centre around what happens when a colony

TABLE 9.

RECONSTRUCTION EXPERIMENT

PROPORTION OF LETHALS RECOVERED

LETHALS	NON-LETHALS	TOTAL	LETHALS RECOVERED AS % OF LETHALS EXPECTED
1	39	40	5.0
1	34	35	5.8
0	40	40	0
0	40	40	0
3	37	40	15
12	28	40	60
9	31	40	45
9	151	160	11.2

starts growth. One possibility is that limited food which results in early conidiation might reduce the time of the growth phase, and possibly eliminate selection. Another possibility is that unlimited food might improve growth conditions and minimise competition between lethal and non-lethal nuclei, and bring about the same effect.

Finally it has been found that the relative proportions of nuclear types in a heterokaryon bear only a very loose relationship to the proportion in the original inoculum (Pittenger and Brawner 1961, Brown unpublished).

Unless this selection can be estimated, data on sector sizes, and even the detection of mosaics may never be very reliable.

(b) 2-chloroethyl methane sulphonate (CB1506)

Previous experiments by Auerbach (1960) using this chemical on Atwood's strain have shown it to be highly effective as a mutagen when it was mixed with the growth medium but ineffective when used in overnight treatment of a suspension of conidia. However, when applied by Kølmark to a suspension of adenineless conidia it was highly effective in producing adenine reversions (Kølmark in Westergaard).

One possible explanation of the ineffictiveness of the mutagen could be that a change in nuclear number occured during the 12 hours of treatment necessary for the effective action of this chemical. The effect of a change in nuclear number brought about by division of the nuclei during treatment, would be to increase the number of nuclei per conidium. Such an increase would result in more conidia conatining >1 methionineless nucleus, and thus lead to a higher proportion of negative results in the detection of recessive lethals as explained in Part I.

Therefore a preliminary investigation of possible changes in nuclear number during prolonged treatment was made. This was determined by the use of ultraviolet light and Huebschman's technique previously described. Before the ultraviolet experiment could be carried out, the effective dose range had to be determined.

1. ULTRAVIOLET DOSE-EFFECT CURVE FOR KILLING AND MUTATION.

Previous workers (Auerbach, Atwood) had found that a high mutation frequency is obtained at approximately 1% survival. So an experiment was done to determine the conditions under which a conidial suspension irradiated with the ultraviolet light source to be used, gave this survival

Conidia were irradiated as described on page 10. Samples were removed at appropriate intervals and immediately diluted for subsequent plating to determine survival levels. This method requires interruption of the irradiation for the time

necessary to remove samples.

The results are given in Table 10. Those for Experiment I indicate that after 6 minutes treatment with ultraviolet light, a survival of 1.5% is obtained. It was considered probable that conidia shielding each other at the suspension density of 3×10^6 per ml. used, could lower the level of mutation induced to an undesirable level. Therefore the experiment was repeated at the lower suspension density of 1.115×10^6 per ml and the results are given in Experiment II, Table 10. The mutation frequency was determined simultaneously with this experiment for conidia treated for 5 minutes with ultraviolet light. After subtracting the lethals found in the controls, 28% recessive lethals were obtained at a survival of 2.9%. It was decided that 6 minutes treatment at the lower suspension density would be used.

Graph II is a plot of the results obtained in Experiment I. The graph secured is similar to that obtained by Goodman for a micro-conidial strain of Neurospora crassa treated with ultraviolet light. He interprets the graph obtained as due to both cytoplasmic and nuclear inactivation. At low doses of irradiation, conidia are inactivated by inactivation of nuclei, or by induction of recessive lethal mutations. At higher doses, cytoplasmic inactivation isa added to the process of nuclear inactivation.

2. TO DETERMINE IF THE NUCLEI DIVIDE DURING PROLONGED SHAKING IN WATER.

If the nuclei divide during prolonged shaking in water, the increase in the number of nuclei per conidium should be detected by sampling at appropriate intervals and determining the frequency distribution of nuclei in the conidia. Also, if the samples removed are treated with ultraviolet light and the induced recessive lethals determined by Atwood's method, it would be expected that nuclear division would result in a fall in the lethals detected. This is due to the fact that with an increase in nuclear

TABLE 10

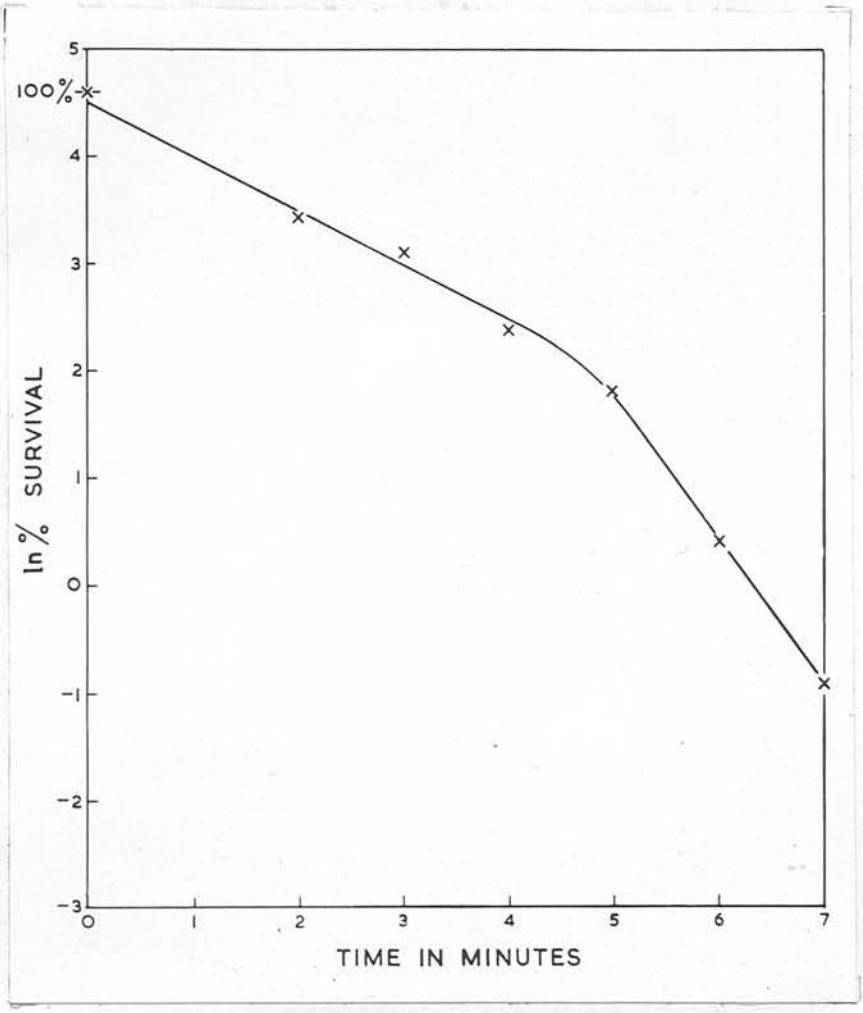
THE EFFECT OF ULTRAVIOLET LIGHT ON (a) Killing (b) Mutation

(a)

TIME OF TREATMENT IN MINUTES	0	2	3	4	5	6	7	SUSPENSION DENSITY / ml.	TREATED VOLUME ml.
% SURVIVAL EXPERIMENT I	100	30.9	22.5	14.1	6.1	1.5	0.4	3×10^6	25
II			37.0		2.9			1.115×10^6	10

(b) ΣEXPERIMENT II

	% SURVIVAL	NUMBER OF COLONIES PUNCHED	NUMBER OF LETHALS	NUMBER OF NON-LETHALS	% LETHALS
Control	100	236	7	229	2.5
Treated for 5 mins	2.9	325	99	226	30.5



GRAPH II:- The effect of ultraviolet light on the survival of the heterokaryon.

number fewer lethals are detectable because of the consequent increase in conidia with more than one methionineless nucleus.

Therefore conidial suspensions were shaken in separate flasks for 0, 3, 6, 9, 12 and 15 hours. At the end of each of these intervals samples were taken for treatment with ultraviolet light as described on page 10. Nuclear counts were done at 0 and 15 hours in Experiment I. The results for two experiments are given in Table 11.

This table shows that the % survival tended to drop with time, especially in Experiment 2. This is probably due to sampling error.

The heterogeneity χ^2 for the combined frequencies after different times of shaking is 6.55 for 5 degrees of freedom. This gives a 20-30% chance that the figures do not represent true heterogeneity.

Since in these two experiments, the induced frequency of recessive lethals did not decrease significantly with prolonged shaking in water, there cannot have been any marked increase in nuclear number. This agrees with the cytological data where the frequency distribution of conidia with various numbers of nuclei is the same at 0 and 15 hours of shaking, allowing for sampling error.

These findings show that the ineffectiveness of CB 1506 on conidial suspensions cannot be attributed to the division of nuclei during the prolonged treatment necessary.

Therefore, an alternative explanation to account for this ineffectiveness on conidial suspensions was investigated. It is possible that mosaics are formed with this mutagen, which would not be scored in a test for recessive lethals. However, in the case of adenine reversions, mosaics will be scored as mutants regardless of sector size since only mutants grow on the minimal

TABLE 11.

(a) THE EFFECT OF SHAKING CONIDIA IN WATER BEFORE U.V.TREATMENT AS MEASURED BY SURVIVAL AND DETECTABLE RECESSIVE LETHALS

U.V.IN MINS	DURATION OF SHAKING IN HOURS	COLONIES COUNTED		% SURVIVAL		COLONIES PUNCHED		LETHALS FOUND		% LETHALS	
		Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
0	0	332	458	100	100	50 50	50	0	0	0	0
0	9	295		88.9		50					
0	15	401	436	120.8	95.1	50	50	0	0	0	0
6	0	72	427	2.3	2.1	100	250	43	63	43	25
6	3	709	316	2.2	1.4	100	50	33	18	33	36
6	6	2648	177	1.5	0.8	100	50	38	18	38	36
6	9	2064	475	1.2	0.9	100	50	38	15	38	30
6	12	1650	286	0.9	0.5	100	50	45	17	45	34
6	15	1718	156	1.9	0.03	100	50	37	13	37	26

(b) THE EFFECT OF SHAKING CONIDIA IN WATER ON THE NUMBER OF NUCLEI PER CONIDIUM (Experiment 1)

DURATION OF SHAKING	NUMBER OF NUCLEI PER CONIDIUM							TOTAL	AVERAGE NUMBER OF NUCLEI PER CONIDIUM
	1	2	3	4	5	6	7		
0	123	282	70	12	5	4	4	500	2.044
15	132	270	72	18	5	3		500	2.006

medium used. Therefore, experiments were done with the aim of trying to detect the presence of mosaics.

Before this , however, Auerbach's experiments on conidial suspensions treated with the chemical, were repeated to see if similar results were obtained.

3. EFFECT OF 2-CHLOROETHYL METHANE SULPHONATE ON ATWOOD'S STRAIN.

This experiment was done in conjunction with Dr. Kølmark who applied the same treatment simultaneously to a suspension of adenineless conidia as described on page 10. This was to ensure that the chemical which had been stored for some time in this laboratory was still effective in the treatment of the adenineless strain.

Conditions of treatment were similar for the two strains and the survival was 59.2% for the adenineless conidia and 12.5% for the arginine/ methionine amycelial strain.

The frequency of reverse mutations to adenine independence was 247 per 10^6 conidia. No recessive lethals were detected in the 253 colonies tested in the arginine / methionine amycelial strain.

Thus the mutagen produces a very high frequency of reverse mutations at the adenine locus. A much higher frequency is expected for Atwood's strain in which forward mutations are being scored at all loci and not at one specific locus.

4. TEST FOR MOSAICISM AFTER TREATMENT WITH 2-CHLOROETHYL METHANE SULPHONATE.

This experiment was done with two important modifications.

(a) Conidia were given the opportunity to replicate during treatment This was done to determine whether replication was necessary to produce mutations since this mutagen is highly effective when applied to the growth medium but not in resting spores. The conditions for

for replication were determined by shaking a conidial suspension in liquid minimal medium, and withdrawing samples at intervals and staining by Huebschman's method as described previously. Two hours was found to be the time required to ensure that cell replication had occured. Therefore 0.4 ml. of liquid minimal medium containing 10% sucrose was added to 3.6 ml. conidial suspension after 10 hours treatment, and also to the control. Both were then allowed to complete the 12 hours specified on page 10.
(b) Improved sampling methods were used for the detection of mosaic lethals. 82 were tested by the method used for nitrous acid under Method 2 b on page 29. However, as isolates for the mosaicism test were taken from methionine supplemented plates on which heterokaryotic and homokaryotic methionineless amycelial conidia are viable, the possibility exists that these two types of conidia could form a heterokaryon. This would result in a heterokaryon with a lethal mutation remaining undetected by Atwood's method.

Therefore, an improvement of the technique by which replicate samples for mosaicism tests were taken from minimal sorbose plates was used. The possibility of homokaryotic methionineless conidia surviving for some time on minimal sorbose medium after plating was tested. This was done to ensure that such conidia do not remain viable long enough to form a heterokaryon with germinating heterokaryotic conidia, as this would result in the same error as in Method 2b. 2ml. liquid minimal medium supplemented with 50 mg/ litre methionine was poured on to minimal sorbose plates previously inoculated with conidia from a non-lethal culture. This was done in triplicate, and the plates incubated at 30°C were examined daily for the presence of amycelial colonies. Amycelial colonies appeared on plates which had been overplated on the first and second days. However, none appeared on plates which had been overplated on the 3rd day onwards. Since sampling for mosaics is done on the 5rd day when there are no

viable amycelial colonies, this indicates that viable amycelial colonies do not constitute a source of possible loss of mosaics.

This new method of sampling from minimal sorbose plates necessitated simultaneous plating on methionine supplemented plates to ensure that only non-lethals were submitted to the test for mosaicism. 33 colonies were tested by this method, 15 - 40 replicates being sampled from each. Two colonies were found to be mosaics the size of the mutant sector being 1/19th and 1/20th of the sample tested.

The possibility that competition in the 'punch tubes' might contribute to the small size of mutant sectors was tested. 45 colonies were grown in large test tubes, and the conidia harvested, filtered through glass wool and the suspension density determined. The conidia were then plated thinly on minimal sorbose plates from which isolates were taken for mosaicism tests, and on methionine sorbose plates for scoring the presence of amycelial colonies to ensure that only non-lethals were submitted to the test. 50 replicate samples were taken from the minimal sorbose plates, and submitted to a test for recessive lethals. Of 45 colonies grown in large test tubes, one was found to be ammosaic. The size of the mutant sector was 1/ 50th of the tested sample.

The results of this experiment are given in Tables 12 a and 12 b. Table 12 a shows the frequency of complete and mosaic lethals. As is shown, no mosaics were found among the 82 tested for mosaicism while sampling was done on methionine sorbose plates. Three mosaics were found among the 78 tested for mosaicism when sampling was done on minimal sorbose plates. This is 3.9% when expressed as a % of the number of colonies tested for mosaicism. However, when expressed as a % of the number of sectors sampled from each original colony, the value obtained does not exceed that expected on the basis of spontaneous mutation.

TABLE 12 a

THE EFFECT OF 2-CHLORO ETHYL METHANE SULPHONATE ON THE FREQUENCIES OF COMPLETE LETHALS AND MOSAIC LETHALS

SERIES	COLONIES COUNTED	% SURVIVAL	COLONIES PUNCHED	% LETHALS	NUMBER TESTED FOR MOSAICS	MOSAICS FOUND
CONTROL						
+ water	631	100	480	0		
+ sucrose	680	100	120	0		
TREATED						
+ water	834	54,4	360	0.5		
+ sucrose	1382	127,0	240	0	82 - 33 -- 45 ---	0 2 1

\- 20 -30 sectors sampled from each original isolate using methionine supplemented plates

-- 15 -40 " " " " " " " " " " and minimal sorbose plates.

--- 35 -50 " " " " " " " " " " " " "

TABLE 12 b

NUCLEAR COUNTS FOR 2-CHLOROETHYL METHANE SULPHONATE EXPERIMENT

(CB 1506)

SERIES	PREVIOUS TREATMENT	NUMBER OF NUCLEI PER CONIDIUM 1	2	3	4	5	6	AVERAGE NUMBER OF NUCLEI PER CONIDIUM
TREATED	Shaken 10 hrs in CB1506 + water	30	55	10	4	1		1.91
	" 12 " " "	26	50	16	5	3		2.09
	" 10 " " " + 2 hrs with sucrose added	4	38	38	12	4	4	2.86
CONTROL	Shaken 10 hrs in water	20	61	12	4	3		2.09
	" 12 " " "	30	48	21	1			1.93
	" 10 " " " + 2 hrs with sucrose added	13	60	19	5	3		2.25

Table 12b gives the result for the nuclear counts determined during the experiment. These show that shaking in water does not affect the distribution of nuclei, whereas shaking in sucrose solution increases the average nuclear number.

The results obtained show that even with improved sampling methods the recovery of the lethal fraction of a mosaic was very low. Therefore it is possible that the frequency of mosaic lethals found is an underestimate, since the majority may remain undetected. This mutagen has also been found to give very small mutant sectors in Drosophila (Auerbach unpublished). If this is also the case in Neurospora, then the probability of detecting mosaics in a system in which selection operates against the mutant fraction of a mosaic is very low.

These results lead to the investigation of an alternative strain.

PART III. ALTERNATIVE STRAIN FOR ATWOOD'S METHOD

In the ragged histidineless nicotinic acidless/ adenineless heterokaryon, it is possible to distinguish the ragged and adenineless morphology by the use of the appropriate media. The best conditions for doing this was determined with the aim of using this as an alternative strain for Atwood's method.

The heterokaryon was grown on sorbose medium containing 0.1 gm sucrose, 0.2 gms fructose or 0.1 gm glucose per 100 ml medium to determine on which medium the ragged morpholory was best manifested. The medium was supplemented with 100 mg/litre histidine, 10 mg/ litre nicotinic acid and various concentrations of adenine - 100, 5, 2, 1, 0.75, 0.5 and 0.25 all in mg/litre. The autoclaving time was carefully regulated at 15 lbs pressure for 15 minutes as this affects the growth characteristics(deSerres, personal communication).

The best results were obtained on the following medium:- 0.1 gm sucrose, 1.0 gm sorbose, 2.0 gm agar per 100 ml media, supplemented with 100 mg/ litre histidine, 10 mg/ litre nicotinic acid and 0.5 mg/ litre adenine.

The components of the heterokaryon were best recognised when plating was done thinly, and were detected after 2 days incubation at 30°C (See Plate 2).

DISCUSSION.

The use of Atwood's method for the detection of complete lethals and its adaptation for the detection of mosaic lethals has revealed some limitations in technique and strain which must be considered.

The selection of conditions for growth under which the maximum number of heterokaryotic conidia contain one amycelial nucleus minimises the loss of scoreable lethals. Also, by means of the corrections made for the presence of more than one methioninelessnucleus per conidium, an estimate of this loss of lethals has been made and taken into account. Such lethals would be scored as mosaics, since the existence of a lethal in more than one methioninelessamycelial nucleus is most likely to result in complementation, and amycelial colonies will be formed.

The ease of detection of a mosaic varies directly with the size of the mutant sector. If a mutant sector is large, fewer replicate samples will be needed for its detection than when it is small. Therefore, a mosaic with a very small mutant sector may be erroneously classified as a non-lethal if the sampling is inadequate.

However, even with adequate sampling the system is still subject to limitations. In the reconstruction experiments selection against the nuclei carrying a lethal mutation was shown to be a possible cause of small mutant sectors. This results in an underestimate of mutant sector size in a mosaic.

Thus it would be advantageous if selection against nuclei carrying a lethal mutation could be reduced or eliminated by use of the alternative strain ragged histidinelessnicotinic acidless / adeninelessheterokaryon. Themarker,ragged, is not as drastic a modification as amycelial, thus might not be so prone to selection when associated with a lethal in the same nucleus. Thus sector size data might be secured which could be interpreted in terms of mutagen action, and the great advantages of Atwood's method with regard to rapid sampling by the 'punching' technique and rapid plating by the 'squirting' technique could usefully be applied to detection of mosaics after treatment with different mutagens.

An alternative strain is particularly important in the case of the mutagen 2-chloroethyl methane sulphonate. A heterokaryon in which both components carry a reversible adenine mutant, would allow the same conidia to be used in tests for recessive lethals and for adenine-reversions at the same time. This would be useful in clarifying the action of this mutagen which is known to give adenine reversions but no detectable recessive lethals or mosaics when conidial suspensions are treated.

The results obtained, although subject to errors resulting from possible undetected mosaics and underestimates of mutant sector sizes, do show certain trends. The proportion of mosaics among mutations decreased from nitrous acid to ultraviolet light to X-rays. The correction factor applied to obtain the true value for mosaic frequency results in overcorrections when a mutagen acts by nuclear killing. This would increase the estimate of mosaic frequency for X-rays and possibly for ultraviolet light, but would probably not reverse the trend shown. This trend is consistent with that found in Drosophila and maize in which the number of mosaic mutations is smaller for X-rays than for chemical mutagens and ultraviolet light. Mosaics form 50% or more of all visible mutations in the F_1 of adult males treated with mustard gas (Auerbach 1945b) as compared with 10-15% of X-ray mutants (Auerbach 1949). Also the amount of fractional to whole body mutation found among the visible mutations of treated males was 7%, 30%, 52% and 67% for X-rays, 2,5 bis ethyleneiminohydroquinone, phenylalanine mustard and dimethyl myleran respectively (Browning and Altenberg 1961). In maize pollen, the proportion of fractionals found is much larger after ultraviolet treatment than for X-rays among phenotypically identified chromosomal derangements in the endosperm (Stadler 1941).

However, the reconstruction experiments show that there is selection against the mutant, thus the sector sizes found cannot be used for drawing conclusions about mechanism of mutation. However, since both complete and mosaic lethals were

detected, some consideration of the possible mechanisms will be attempted.

Two types of mechanisms for the formation of complete lethal mutation in double stranded genetic material will be considered. Firstly, mechanisms which cause non-complementing changes in both strands, and secondly mechanisms which reduce the operative genetic material to one strand.

Within the first type of mechanism there is the case of non-complementing mutations occuring independently in the same cistron of both strands of the DNA double helix. The probability of this occuring is equal to the square of the probability of the occurence of a mutation in that cistron. Since the whole genome is being tested here, such double mutations may be assumed to make a negligible contribution. For the purposes of Atwood's method, this mechanism may be regarded as operationally identical to that suggested by Muller et al 1961 when considering X-ray induced mutations. He proposed a mechanism which he called 'rotational' substitution' where the purine-pyrimidine base pair has both its bonds to the sugars in the main helical backbones broken by the mutagen. The hydrogen bonds remain intact and keep the pair in their correct position relative to each other. They are thus left free to rotate within the chain and have an even chance of rejoining to the wrong sugars, the purine where the pyrimidine was originally joined, and the pyrimidine where the purine was. In this mechanism a double mutation is brought about by a single event, the single ionizing electron breaking both bonds. This mechanism is supported by the linear relationship between mutation and ionizations, and its independence of time and wavelength (Timofeeff-Ressovsky 1930). Also the fact that minute rearrangements in Drosophila caused by X-rays follow a linear dose-effect curve (Muller 1940, Frye 1957) shows that it is possible for a single ionizing electron to affect two portions of a chromosome which are in close proximity.

This mechanism may also be used to explain complete mutations induced by ultraviolet light. It is possible that ultraviolet excitation may affect both strands in the same region of the chromosome creating similar functional effects in the same cistron of both strands, although the actual changes in the genetic material may not be identical.

However, this mechanism would have to be greatly modified to cover complete lethals induced by nitrous acid. Vielmetter and Schuster (1960) have shown in phage T_2 that the deamination of guanine is responsible for inactivation alone, whereas the deamination of adenine and cytosine causes mutation as well. These deaminations change the hydrogen bonding structure in adenine and cytosine, and it is possible that this disarrangement of the hydrogen bonding pattern may create instability which may then be rectified if either base is replaced by a base with a hydrogen bonding pattern complementary to the remaining base. If the normal base is the one replaced, the remaining deaminated base will induce the incorporation of a new base with a hydrogen bonding pattern complementary to its own, namely the mutated form of the remaining base. Consequently, a complementary mutation will have occured. The other possibility constitutes a recovery. This mechanism will also give a linear dose effect curve, since it requires a single initiating event.

In the second type of mechanism in which the operative genetic material is reduced to a single copy, Tessman (1959) for phage T_4 suggests that parts of the chromosomes contain only a single strand, and it is these regions which produce complete mutations. Such a mechanism would give a linear dose effect curve, which has been found to be the case for phage T_4 (Vielmetter, Schuster and Wieder 1959). Alternatively, mutation may occur in one strand and the other strand may fail to transmit the genetic information. This was also proposed by Tessman. Complete lethals induced by X-rays and ultraviolet light may be explained by this mechanism, by proposing that a single ionization or excitation produces a recessive lethal in one strand and an effect in the

other which arrests replication. This mechanism may only be used to explain complete lethals induced by nitrous acid if either an exponential dose effect curve is found, or one assumes that the processes which cause inactivation and mutation are sufficiently different that only one is limiting throughout the dose range.

The acceptance of the double nature of the genetic material helps considerably in explaining the existence of mosaics. However, it poses further problems of determining which of the mechanisms now possible are responsible for their formation. Here again there are two main types of mechanisms which may produce mosaics. Firstly, a mutation in one strand of a double stranded gene, and secondly an error in replication. The relative size of the mutant fraction may enable one to decide between them, if selection against nuclei carrying a lethal could be eliminated.

In the first mechanism, one strand will be affected and the other remain unchanged. Thus since each chromosome strand guides the assemblage of its own complementary daugheter strand (Taylor, Woods and Hughes 1958) there should be a half mutant and a half non-mutant. In the second mechanism, except in the unlikely event of non-complementing mutations taking place in each strand, only one quarter would be mutant. These two values for the mutant sectors are the maximum values, and assume in the first mechanism that the effect was immediate, and in the second mechanism that the error occured at the first replication.

However, if there is a delay in either the realisation of what in fact was a potential mutation, or a delay in terms of replications before the error occured, smaller mutant sectors would be expected and the size of the mutant sector would vary inversly with the length of the delay. Also, the mutation may be induced after the withdrawal of the mutagen. This would result if either residual mutagen, or a mutagenic derivative produced during treatment remains in the cytoplasm.

A tendency for a delayed effect has mainly been

observed after treatment with chemical mutagens and ultraviolet light, as opposed to X-rays. One source of evidence for a delayed effect comes from mosaicism arising much later than the time of treatment.

The best evidence for the delayed occurence of a mosaic mutation comes from Drosophila . Gonadic mosaics among the progeny of mustard gas treated males have given progeny which exhibit gonadial mosaicism for the same mutation that occured in the parents (Auerbach 1949). In this case a whole generation, representing about 30 divisions, has elapsed between treatment and mutation. Here it is assumed that the chemical reacts with the genetic material producing an effect which is not an immediate mutation, but results in increasing the probability of an error in replication. Such a change would give rise to a mosaic.

Therefore the consequences of a delayed effect must be taken into account, particularly for the mutagens ultraviolet light and nitrous acid. If the rate of nuclear division for lethal and non-lethal nuclei are the same, then 50% recovery of each is expected. However, if any delay or retardation occurs in the division of the mutant nucleus, distortion of the expected ratio would result (Pittenger and Brawner 1961). In the case of the delayed error in replication, the likelihood of an error is expected to increase with the number of replications. Also as the number of replications between the start of replication and the occurence of the error increases, the proportion of the mutant sectors gets smaller and thus become more difficult to detect. In the case of a mutation occuring in one strand only, there will only be one potential mutation regardless of how many replications may take place. Thus the probability of realisation of the mutation is always the same regardless of how many divisions take place.

Thus there should be equal numbers of mosaics with any given sector size. This last result hasbeen obtained in phage T_4 by Greena and Krieg (1961) for the mutagen ethyl methane sulphonate.

Besides delayed mutation, mosaicism can also be attributed to the segregation of mutated and non-mutated strands in a multistranded chromosome. These cannot be distinguished experimentally.

The distinction between potential and actual mutation is important in all mutation work. Often the metabolic state of the cells after treatment decides whether this 'potential' mutation shall become an actual mutation or undergo restitution. This has been shown in bacteria by Witkin (1959). Here one group comprising mutations from auxotrophy to prototrophy is expressed only when protein synthesis takes place in the treated cells during the first hour after ultraviolet irradiation. If protein synthesis is inhibited the mutations are not expressed. Another group of mutations from streptomycin sensitivity to streptomycin resistance is expressed irrespective of whether protein synthesis takes place after irradiation or not. It is possible that the inconclusive results obtained with the mutagen 2-chloro ethyl methane sulphonate, amy be due to the conditions for realisation of a potential mutation not being met.

One must bear in mind, however, the highly complicated biological systems being dealt with. Thus fundamental genetic mechanisms are quite likely to differ with the organism, and interpretations of the mutation process must essentially remain somewhat obscure.

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ADDENDUM

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SUMMARY.

Atwood's method for the detection of recessive lethal mutation in Neurospora crassa and its adaptation for the detection of mosaic lethal mutation, was investigated with the aim of determining the best conditions under which these two types of mutations could be detected.

The strain used was a balanced heterokaryon between arginineless and methionineless amycelial nuclei. Mutations are scored only in the methionineless amycelial nuclei, where the presence of the gene amycelial, which gives a morphological modification, greatly facilitates scoring.

The presence of more than one methionineless amycelial nucleus per heterokaryotic conidium results in the observed values for lethal and mosaic lethal mutations being not the true values. This effect was minimised by selecting conditions under which the majority of conidia contained two nuclei, and the observed results were corrected for the effect of conidia with more than one methionineless amycelial nucleus. As the corrections made depend on whether or not the mutagen acts by nuclear killing, this was also taken into account.

The proportion of mosaics among mutations was found to decrease from nitrous acid, to ultraviolet light, to X-rays. In all cases the lethal sector of a mosaic was much smaller than one half, the majority comprising 20% or less. As the reconstruction experiments showed, this was due to selection against the lethal sectors. This would lead to an underestimate of mutant sector size, and also to the non-detection of mosaics with very small mutant sectors. This might possibly be the cause for the inconclusive results obtained with 2-chloroethyl methane sulphonate.

The advantages of an alternative strain in which this selection might not occur is discussed.

Possible mechanisms for the origin of complete and mosaic lethal mutations are proposed.