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Mutagenic action of nitrous acid on *Aspergillus nidulans*

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1. INTRODUCTION

In recent years the mutagenic action of nitrous acid has attracted considerable attention. Nitrous acid is known to deaminate nucleic acid bases, changing guanine to xanthine, adenine to hypoxanthine and cytosine to uracil. It has been widely assumed that its mutagenic action results from these alterations of the bases (Schuster & Schramm, 1958; Gierer & Mundry, 1958; Kaudewitz, 1959*a*, *b*; Vielmetter & Wieder, 1959; Tessman, 1959). Direct support for this view comes from the work of Litman & Ephrussi-Taylor (1959) who have shown that nitrous acid can cause mutations in the transforming DNA. Among the more striking properties of nitrous acid as a mutagen are its marked preference for specific mutational sites ('hot spots') within a cistron and a characteristic pattern of inducibility with respect to forward and backward mutations at particular sites (Freese, 1959; Benzer, 1961). The kinetics of nitrous acid-induced mutation rates have also been interpreted in terms of its action on a two-stranded DNA molecule (Kaudewitz, 1959*a*, *b*).

The present paper describes the action of nitrous acid on *Aspergillus nidulans* where it has proved to be an effective mutagenic and killing agent. It may be pointed out that the mutagenic action of nitrous acid was first reported over twenty years ago by Steinberg & Thom (1940*a*, *b*), who obtained morphological mutants in *A. niger* and *A. amstelodami* by growing these species in a medium containing 0·2% $NaNO_2$. This work seems to have been overlooked by some of the recent investigators. It is interesting to note that the mutagenic action of nitrous acid was, at that time, ascribed to the deamination of gene-proteins.

2. MATERIAL AND METHODS

The techniques employed in the present work are those in current use in the genetics of *A. nidulans*. For these and for the origin of strains, reference may be made to Pontecorvo, Roper, Hemmons, Macdonald & Bufton (1953).

Method of treatment

The conidia were harvested from 5–6 days old cultures, grown on slopes by pouring 10 ml. of $\frac{1}{1000}$ calzolene oil in saline into the culture tubes and shaking vigorously until most of the conidia went into suspension. The suspension was filtered through sterile cotton wool to remove the mycelial debris and the conidial chains were broken up by repeatedly sucking the suspension up and down through a Pasteur pipette.

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The suspension was centrifuged and the conidia washed and resuspended in 0·1M acetic acid-sodium acetate buffer at pH 4·4. Freshly prepared 0·5 M solution of $NaNO_2$ was added to give a final concentration of 0·017 M.

Treatment was carried out in a water bath at 37° C. on a suspension of about 4×10^8 conidia in 20 ml. of liquid made up as follows:

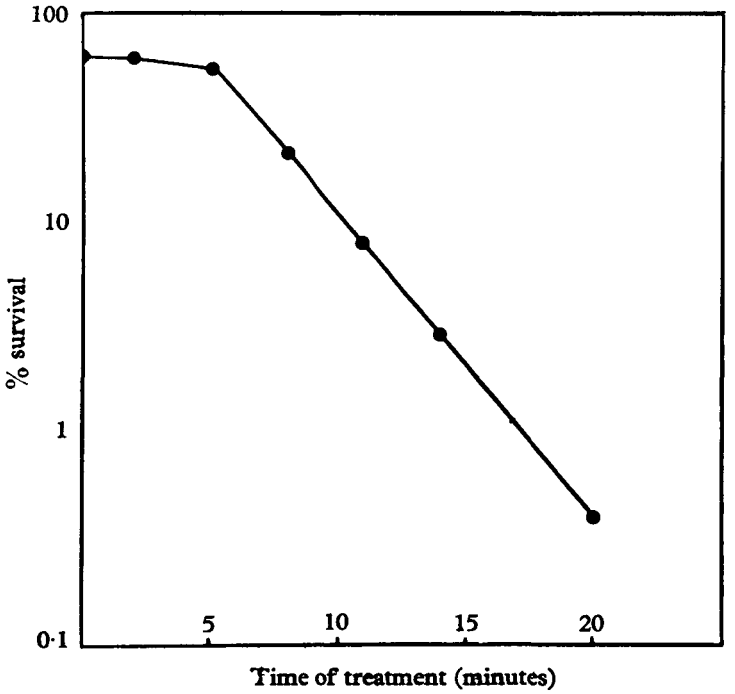
	ml.
0·5 M $NaNO_2$	0·66
0·1 M acetate buffer, pH 4·4	19·33
Total volume	20

The treatment could be stopped at required intervals of time by transferring samples to pH 7 phosphate buffer. When large numbers of conidia were needed, the treatment suspension was neutralized with M/10 NaOH and the conidia were washed by successive centrifugation and resuspension in saline. The controls were handled in the same way except that $NaNO_2$ was not added.

3. RESULTS

Survival rate

The survival rate of a *meth ; bi* strain (requiring methionine and biotin) on treatment with nitrous acid is shown in Text fig. 1. Under the usual conditions of treatment more than 90% of the conidia were killed by about 14 minutes. The survival curve is exponential with a distinct shoulder near the origin.



Text fig. 1. Survival of the conidia of *A. nidulans* strain $meth_1 bi_1$ after treatment with 0·017 M $NaNO_2$ at pH 4·4.

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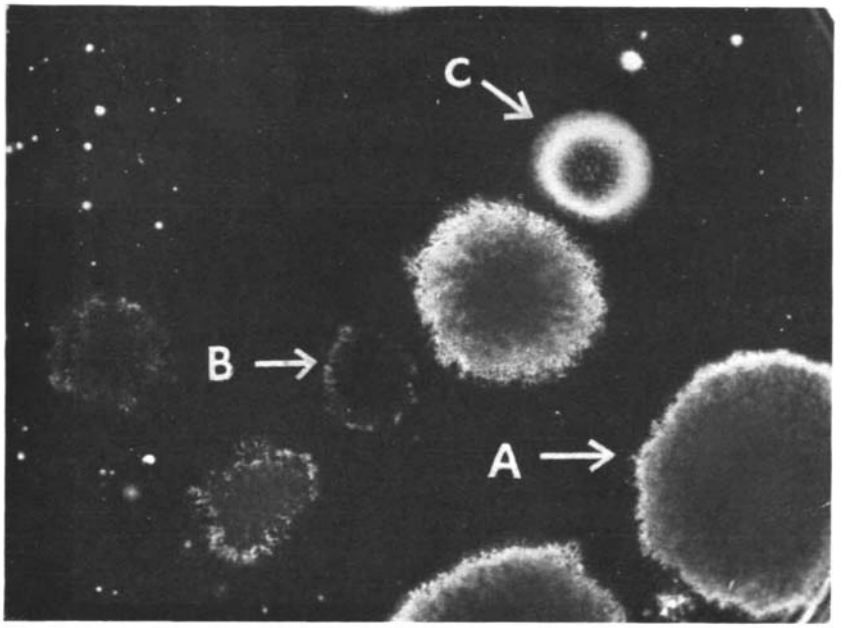

Fig. 1. Suppressors of methionine requirement: A = *SuA*; *B* = *suB*; *C* = *SuC*.

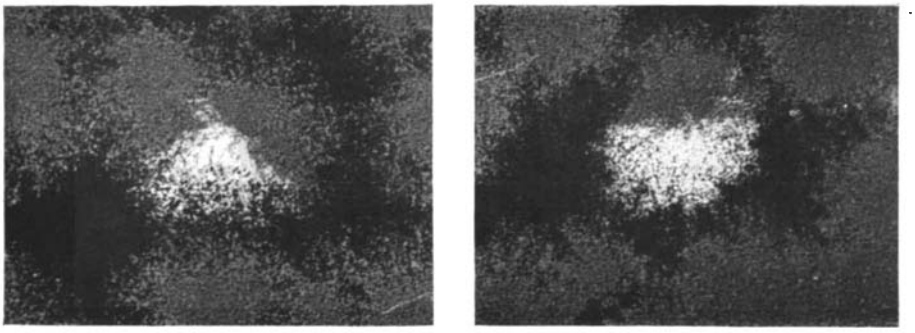

Fig. 2. White mutant sectors produced by nitrous acid.

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Mutagenic effect

In preliminary experiments, the treated conidia were plated on complete medium (C.M.) to give about 100 colonies per dish. These colonies were tested for auxotrophic mutations by replica plating (Roberts, 1959). After 12 minutes of treatment about 0·2% of the colonies from the treated conidia were auxotrophs. Induced mutation rates were, then, investigated by means of the back-mutation screening system used in micro-organisms (Jensen, Kirk, Kölmark & Westergaard, 1951).

The system for estimating induced mutation rates

To obtain the dose-effect curves for induced mutation rates, an assay system based on the reversion of a methionine requiring strain ($meth_1$) to methionine independence was employed. This system has been devised by Dr L. J. Lilly and I am indebted to her for making available to me some of her unpublished results.

Conidia of the $meth_1$ strain when plated on a medium lacking methionine give origins with a low frequency to a few methionine-independent colonies. The revertants consist of several morphologically distinct types and involve suppressor mutations which are unlinked to $meth_1$ (Lilly, unpublished). Three of the revertant types which are clearly distinguishable from each other were chosen to investigate the mutation rates. These revertant types have been designated as *Su* $meth_1A$, *su* $meth_1B$ and *Su* $meth_1C$, abbreviated in the present paper as *SuA*, *suB* and *SuC*. On medium without methionine, *SuA* forms thinly growing colonies, *suB* produces an intense brown pigment, while *SuC* gives thick colonies with hyaline edges (Plate I, Fig. 1). The system offered two advantages. Firstly, as the reversion rates are high —of the order of 10^{-5}—adequate numbers of revertants could be obtained on a relatively small number of dishes. Secondly, as the different suppressors can be scored separately, it is possible to follow their respective mutation rates in the same experiment.

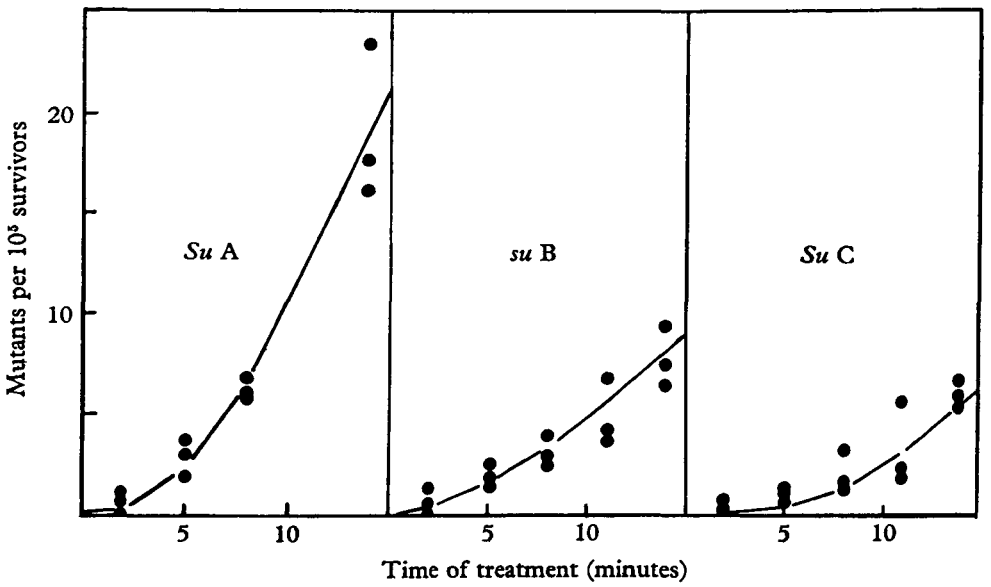
In order to determine the dominance relationship of the suppressors, balanced heterokaryons and diploids homozygous for $meth_1$ but heterozygous for one of the suppressors, were synthesized. These were repeatedly tested for growth on medium without methionine. *SuA* and *SuC* were partially dominant while *suB* was recessive. The partial dominance of *SuA* and *SuC* makes difficult a clear-cut determination of their allelic relationship by means of complementation tests. However, heterokaryons and diploids, homozygous for $meth_1$ and with different two by two *trans* combinations of the three suppressors, were synthesized and their growth on methionineless medium was compared with that of diploids homozygous for each of the suppressors. While the relationship of the partially dominant *SuA* and *SuC* could not be determined in this way, *suB* complemented each of the others. Attempts to make crosses between different suppressors did not succeed, and their location on the map has not been determined so far.

Induced mutation rates

The conidia of the *bi* $meth_1$ strain were treated with nitrous acid according to the method described earlier. Samples were removed from the treated suspension to

phosphate buffer at pH 7 after different lengths of treatment and screened for suppressors of methionine requirement by plating on minimal medium supplemented with biotin. Viable counts for each sample were obtained by plating suitable dilutions of the same treated suspensions on medium supplemented with methionine and biotin.

The data from three similar experiments are presented in Table 1 and the dose-effect curves for the three suppressors are shown in Text-fig. 2. In the calculation of the mutation rates, the background rates obtained by plating untreated conidia



Text fig. 2. Mutation rates of three different suppressors of methionine requirement in *A. nidulans* after treatment with 0·017 M $NaNO_2$ at pH 4·4:

$$SuA,\ y = 0{\cdot}1256 - 0{\cdot}018x + 0{\cdot}0935x^2$$
$$suB,\ y = 0{\cdot}167 + 0{\cdot}1841x + 0{\cdot}025x^2$$

have been deducted. It will be seen that the increase in mutation rates is, in some cases, over twenty-fold. At low rates of killing (see expt. 3, Table 1) this involves an absolute increase in the numbers of mutants and therefore—at least at these lower rates—cannot be accounted for by a selection of the mutants by nitrous acid treatment.

The dose-effect curves were tested for linearity by analysis of variance. The curves for *SuA* and *suB* deviate significantly fron linearity ($P = < 0{\cdot}05$) but the deviation in case of *SuC* is not significant ($P = 0{\cdot}2$). The slope of the curve *A* is higher than that of *B*. The closest-fitting regressions for *A* and *B* are given in Text-fig. 2.

To find out whether the departure from linearity in case of *SuA* and *suB* could be accounted for by a differential survival of the mutant conidia, the following reconstruction experiment was carried out. Conidia of a *y meth*$_1$ *SuA bi* strain—with

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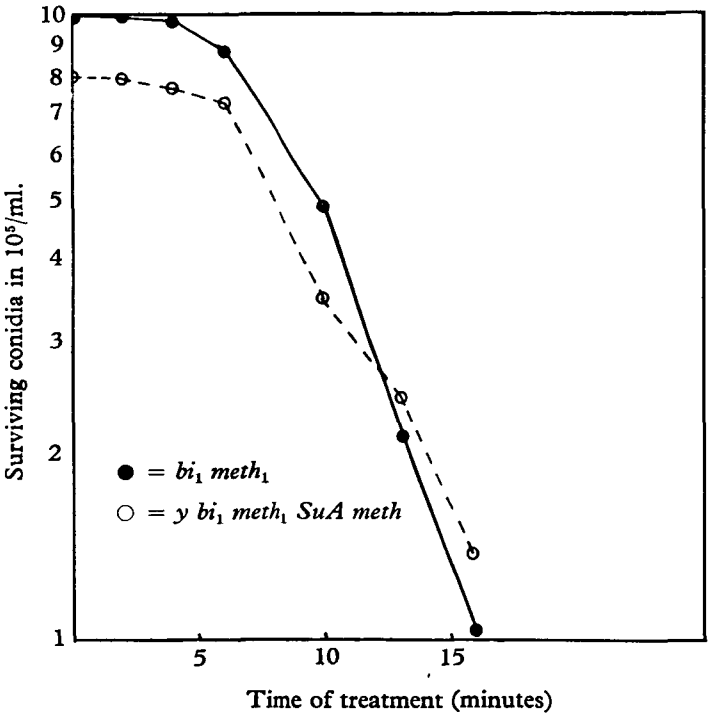
Table 1. *Effect of nitrous acid on reversion of* $meth_1$ *strain to methionine independence* (*induced frequencies have been corrected for the background mutations*)

Time of treatment	Expt. no.	Number of conidia plated	Number of surviving conidia	Per cent survival	Number of revertants			Frequency of revertants × 10^5		
					SuA	*suB*	*SuC*	*SuA*	*suB*	*SuC*
Control	1	9×10^6	6×10^6	66·6	17	9	12	0·28	0·15	0·2
(untreated)	2	9×10^6	6×10^6	66·6	19	15	30	0·31	0·25	0·5
	3	$4·5 \times 10^6$	$3·8 \times 10^6$	62·2	8	16	3	0·29	0·57	0·11
2 minutes	1	9×10^6	$5·5 \times 10^6$	61·1	10	6	2	0·0	0·0	0·0
	2	9×10^6	$6·2 \times 10^6$	69·0	103	103	52	1·29	1·35	0·33
	3	$4·5 \times 10^6$	$3·0 \times 10^6$	68·0	31	31	10	0·72	0·44	0·22
5 minutes	1	9×10^6	$5·0 \times 10^6$	55·5	104	94	74	1·80	1·68	1·33
	2	9×10^6	$3·8 \times 10^6$	42·0	153	102	40	3·69	2·45	0·55
	3	$4·5 \times 10^6$	$3·0 \times 10^6$	66·0	97	62	34	2·97	1·51	1·03
8 minutes	1	9×10^6	$1·9 \times 10^6$	21·1	115	60	66	5·7	2·92	3·28
	2	9×10^6	$2·6 \times 10^6$	29·2	189	107	51	6·79	3·81	1·40
	3	$4·5 \times 10^6$	$2·5 \times 10^6$	56·0	157	73	40	5·94	2·32	1·47
11 minutes	1	$1·8 \times 10^7$	$1·4 \times 10^6$	7·7	146	100	84	9·82	6·70	5·68
	2	$1·8 \times 10^7$	$3·1 \times 10^6$	17·2	298	119	70	9·29	3·55	1·70
	3	$4·5 \times 10^6$	$2·0 \times 10^6$	44·0	198	91	43	9·71	4·02	2·07
14 minutes	1	$1·8 \times 10^7$	$5·0 \times 10^5$	2·7	83	34	35	16·12	6·50	6·75
	2	$1·8 \times 10^7$	$1·6 \times 10^6$	8·7	284	123	102	17·59	7·49	5·90
	3	$4·5 \times 10^6$	$1·2 \times 10^6$	26·0	282	121	64	23·81	9·46	5·36

yellow conidia—and a *meth*$_1$ *bi* strain—with green conidia—were mixed in approximately equal proportions and treated with nitrous acid. Samples were taken out at regular intervals and plated on minimal medium (M.M.) supplemented with



Text fig. 3. A comparison of the sensitivity of the conidia of two strains of *A. nidulans* to nitrous acid treatment.

methionine and biotin. The yellow and green colonies among the survivors were counted separately and the survival curves for the two strains were compared. The results are shown in Text-fig. 3. The difference in the regression coefficients of the two curves is not significant and is unlikely to account for the observed departure from linearity.

Induction of fractional mutations by nitrous acid

It was observed in preliminary experiments that among the colonies obtained from conidia of green (wild type) strains treated with nitrous acid there was a high incidence of mutations to white (*w*) and yellow (*y*) conidia. A large proportion of these colour mutants arose as yellow or white sectors in green colonies. Since the overwhelming majority of the colonies originated from single conidia which are uninucleate and haploid, such sectored mutants could have arisen in one of two ways: (1) a bi- or multi-stranded structure of the genetic material in the nucleus of the conidia; or (2) a delayed action of the mutagen and replication of the genetic material before the occurrence of the mutation. The sectored or the fractional

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mutations were further investigated in an attempt to distinguish between the two possibilities. The strain used for this experiment was one with green conidia, requiring biotin (symbol: *bi*).

In order to exclude the obvious possibility that nitrous acid was being carried over from the treatment mixture to the dishes and causing mutations during the growth of a colony, the conidia were washed repeatedly after treatment by successive centrifugation and resuspension. This did not appear to affect the yield of sectored mutations. The possibility, however, remains that the mutagen is carried over in an unwashable cell-bound state.

The treated conidia of strain *bi* were plated on complete medium to give about 50–100 colonies per dish. The dishes were scored for *w* and *y* mutants after 72 hours of incubation. In one series of experiments, conidia treated in acetate buffer were used as controls; in another series of experiments a sample of conidia removed immediately after the addition of $NaNO_2$ was employed as control. The pooled data from several experiments are presented in Table 2. The frequency of *w* and *y* mutants in different experiments varied from 0·08% to 0·3%. About 80% of the mutants on the average were sectored.

Table 2. *Induction of colour mutants by nitrous acid in the* bi *strain*

A. Conidia washed thrice after treatment; conidia treated in buffer used as control; survival about 5%. (Pooled data of three experiments.)

		Colour mutants			
		Sectored		Unsectored	
Population	Number of colonies screened	*w*	*y*	*w*	*y*
Treated for 20 minutes	8208	19	5	3	2
Control	9400	—	—	—	—

B. Treated conidial suspension diluted 1/10,000 without washing. Conidia removed immediately after the addition of $NaNO_2$ used as control; survival about 25%. (Pooled data of eight experiments.)

		Colour mutants			
		Sectored		Unsectored	
Population	Number of colonies screened	*w*	*y*	*w*	*y*
Treated for 12 minutes	23,320	11	4	3	—
Control	30,605	—	—	—	1

The sectored mutants could also be artifacts produced by clustering of two or more conidia, either as a consequence of inefficient dispersion before plating or of actual aggregation after plating. In order to obviate the first possibility, the conidial chains in the suspension were very thoroughly broken up and examined microscopically: the proportion of unbroken chains was never more than 5–10%. Unbroken chains, therefore, could not account for more than a small fraction of the sectored mutants.

Aggregation of conidia could also occur at the time of plating. To check this possibility, in a 'reconstruction' experiment, conidia from a white strain were mixed with those from a green one in a proportion of 1:20 and the mixture was plated to give about 50 colonies per dish. Among over 1000 colonies examined no white sectors were seen. To account for a proportion of sectors as high as that observed in our experiments, clustering would have to be the rule rather than the exception.

The size of the mutant sectors

The sectored mutants, in most cases, originated at the centre of the colony. The sectored colonies could be classified according to the size of the mutant fraction. In some cases they were clearly a half or a quarter mutant (Plate I, Fig. 2). In other cases the mutant sector either formed less than a quarter of the colony or formed streaks radiating from the centre. A classification of 18 colour mutants obtained in one experiment is given in Table 3.

Table 3. *Classification of 18 colour mutants obtained by nitrous acid treatment*

	Number	
Size of mutant fraction	*w*	*y*
Whole	3	—
One-half	3	—
One-quarter	4	1
Less than one-quarter	2	2
Indeterminate	2	1
Total	14	4

'Double screening' experiments

In order to obtain more information about the origin of the sectored mutants, a 'double-screening' experiment, similar to the experiment of Witkin on bacteria (Witkin, 1951) was carried out. It consisted in scoring the incidence of sectored colour mutants among colonies which had been selected for the presence of another independent mutation. It was expected that if the sectors arose mainly as a result of the delayed action of the mutagen, double screening would not affect their proportion among all colour mutants. On the other hand, if the sectors originated either exclusively or partly as a result of nuclear segregation after the occurrence of the mutation, their numbers and proportions among colour mutants might be affected. The second possibility could be envisaged if, at the time of the treatment, more than one genetic strand were present. Selective plating would be expected to eliminate those strands which did not carry the selected mutation and thus reduce the probability of sectoring for any other mutation.

The suppression of methionine requirement was used as a selective system. Conidia of the *bi meth*$_1$ strain were treated with nitrous acid for 10 minutes and plated on M.M. supplemented with biotin and on C.M. The proportion of sectored to non-sectored white and yellow mutants among the *SuA* colonies was compared with that

among the colonies grown from the same treated suspension on C.M. (*i.e.* not selected for *SuA*). The results are presented in Table 4. It will be noticed that selective plating for *SuA* does not eliminate the sectored mutants. However, their proportion is reduced. Among the unselected colonies the ratio of sectored to unsectored is 19 : 4 while among the *SuA* colonies it is 7 : 10. The 2×2 contingency χ^2, testing agreement of the two proportions, is 16·9 with a probability of less than 1%.

The appearance of some sectored mutants among the *SuA* colonies also shows that these sectors are not artifacts due to a crowding or crumpling of the conidia. As the frequency of *SuA* mutations even after treatment with nitrous acid is of the order of 10^{-4}, the probability of simultaneous mutations in two neighbouring conidia is very small. Some of these colonies, especially where the mutant sectors were very small, could also have been sectored for methionine requirement through cross-feeding.

Table 4. *Effect of selective plating on the incidence of sectored mutants after treatment with nitrous acid*

		Plated on C.M.					Plated on M.M. + biotin colonies *SuA*				
			Colour mutants					Colour mutants			
			Sectored		Unsectored			Sectored		Unsectored	
Plating	Per cent survival	Colonies screened	*w*	*y*	*w*	*y*	Colonies screened	*w*	*y*	*w*	*y*
1	27	8,103	4	5	2	1	7,569	1	1	1	2
2	25·6	5,120	4	2	—	—	3,670	2	—	2	2
3	32·2	3,317	—	1	—	—	3,135	2	1	1	1
4	20·2	2,031	2	1	—	1	2,412	—	—	—	1
Total		18,571	10	9	2	2	16,786	5	2	4	6
			19		4			7		10	

In order to determine whether sectored mutations are also produced by U.V., colonies obtained from U.V.-irradiated conidia were screened for colour mutants. Among 35,206 colonies examined 15 were colour mutants, of which 3 were sectored. It appears that although fractional mutations can arise after U.V. irradiation, their proportion is smaller than in the case of nitrous acid-induced mutations.

4. DISCUSSION

Fractional mutations can be caused by the induction of an unstable state in the gene. This might consist of a predisposition to mutate or some form of incipient mutation. As pointed out by Auerbach, the two possibilities are difficult to distinguish and perhaps not exclusive of each other. Both have been included in the concept of premutation (Auerbach, 1951). Recent work on the mutagenic action of nitrous acid on bacteriophages indicates that the occurrence of mixed clones of mutant and non-mutant phage particles, after treatment with nitrous acid, may be

interpreted in terms of the Watson-Crick double-stranded structure of the DNA molecule (Tessman, 1959; Vielmetter & Wieder, 1959). Pratt and Stent (1959) have obtained similar results with 5-bromouracil. They have used the term 'mutant heterozygosity' for this phenomenon.

An interpretation of the present results in precise molecular terms will not be attempted. The data, at present, are not sufficient to justify such speculation. Some implications of the results may, however, be considered in more general terms. The reduction in the proportion of sectored mutants in double screening experiments appears to indicate that at least a part of the sectored colonies arises by segregation from a bi- or multistranded structure. The inference is that, as selection for *SuA* eliminates some of the strands, it decreases the possibility of sectoring for the second mutation. This interpretation, although favoured by the present author, is not the only one possible. It has been pointed out to me by Dr C. Auerbach that the mutation to suppressors, like mutation to *y* or *w*, may be usually delayed by one or two divisions. In this case the screening would select cells that have undergone residual divisions and for that reason are more likely to have already completed a colour mutation.

The dose-effect curves for *SuA* and *suB* are non-linear. Although the curve for *SuC* does not depart significantly from linearity, this may be due to the wider scatter of observations present in this case. *SuC* is also slow-growing and at higher dosages of treatment, when the number of colonies on the dishes is large, some of the *SuC* colonies may be swamped. It will be noticed that the kinetics of mutation rates are apparently not related to dominance or recessiveness of the mutations. Although *SuA* is dominant and *suB* recessive, both give non-linear curves. Any interpretation of non-linear dose-effect curves in terms of the structure of the genetic material is open to serious doubts. The killing and the mutagenic events may not necessarily be identical and there may be a threshold for a part of the killing effect (the shoulder in the survival curve may well reflect such a threshold). In such a case the mutation frequencies expressed in terms of survivors will be exaggerated at higher doses and the dose-effect curve would tend to simulate a multi-hit form.

A noticeable feature of our results is the large difference between the slopes of the dose-effect curves for *SuA* and the other two suppressors. After 14 minutes of treatment the mutation rate to *SuA* is about $2\frac{1}{2}$ times greater than that to *SuB*. This could result from differences in the 'target size' of the suppressors or specific differences in their sensitivity to nitrous acid. Such differential mutability is well-established with regard to specific sites in back-mutation tests (Demerec, 1954; Kölmark, 1953; Kölmark & Westergaard, 1953; Malling *et al.*, 1959). Differential response of genes to mutagens in forward mutation tests, called by Westergaard (1960) 'interlocus specificity', is, at present, a matter of some controversy. It has been reported in barley, yeast and *Drosophila* (Gustafsson *et al.*, 1948, 1949; Heslot, 1960; Fahmy & Fahmy, 1956, 1957, 1959; however, see criticism by Stern, 1957; Auerbach and Woolf, 1960). From the extensive work on the rII cistron in bacteriophage T4 (Benzer, 1961) it is known that the incidence of mutations at a few 'hot spots' may be many times greater than at less mutable sites. The sensitivity of a gene towards

specific mutagens like nitrous acid is likely to be determined, in the main, by its characteristic 'hot spots' and may well be different for different genes. Westergaard (1960) has pointed out that one of the difficulties in detecting inter-locus specificity is the limited resolving power of most forward mutation assay systems. Phenotypically distinguishable suppressor mutations of the kind employed in the present work would seem to offer more suitable material for studying this problem.

SUMMARY

Nitrous acid is shown to be a potent killing and mutagenic agent for *Aspergillus nidulans*. The kinetics of mutation rates induced by nitrous acid are investigated by means of three phenotypically distinguishable suppressors of methionine requirement. The dose-effect curves for two of the suppressors are non-linear. Among *w* and *y* mutants produced by nitrous acid there is a high proportion of sectored mutants. Double screening experiments indicate that at least some of the sectored mutations may be due to the presence of more than one genetic strand at the time of treatment. Two of the suppressor genes respond differentially to nitrous acid treatment.

I wish to thank Professor G. Pontecorvo, F.R.S., for suggesting this problem, and for advice and criticism.

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