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Studies on the inhibition and mutation of *Aspergillus nidulans* by acridines

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1. INTRODUCTION

Acridines have attracted increasing interest as mutagens but so far relatively little attention has been given to acridine-induced genic or chromosomal mutations in fungi. The present paper reports a search for such mutations, and factors influencing their production, in *Aspergillus nidulans*. The system of Lilly (1965) has been used to select revertants at a number of suppressor loci and, to widen the scope of mutant classes which might be detected, acridine-induced morphological variants have also been examined.

The inhibitory actions of acridines on *A. nidulans* are determined by a variety of conditions and the same could well be true for any possible mutagenic effects. The first part of this paper describes studies on the inhibitory effects of a range of acridines with reference to such variables as genotype, environment and stage of life cycle treated. These investigations were undertaken in part to establish a foundation for mutation work.

2. METHODS

General techniques and methods of genetic analysis were those of Pontecorvo, Roper, Hemmons, MacDonald & Bufton (1953). Location of a mutant allele to its linkage group *via* mitotic haploidisation (Forbes, 1959) was facilitated by the p-fluorophenylalanine technique of Morpurgo (1961).

Media. Minimal medium (MM), Czapek-Dox with 2% glucose. Complete medium (CM), a complex medium with yeast extract, hydrolysed casein, hydrolysed nucleic acid, vitamins, etc. Solid media contained 2% agar. Buffers (final concentrations): M/20 citrate for pH 10, M/100 phosphate for pH7, M/200 borate for pH3.

Acridines. From the following sources: acriflavine (A) (a mixture of 1/3 proflavine and 2/3 proflavine methochloride), proflavine hydrochloride (P), British Drug Houses; 5-amino acridine hydrochloride (5A), acridine hydrochloride (AC), Koch-Light; acridine yellow (AY), coriphosphine, atebrin (AT), acridine orange (AO), G. T. Gurr.

Organisms. Strains were taken from laboratory stocks maintained on CM. Mutant alleles used in this work are described by Pontecorvo *et al.* (1953), Roper & Käfer (1957) and Warr & Roper (1965). Those of main importance were: *y*,

yellow conidia; *bi*1, *meth*1, *ribo*1 and *ribo*2, *nic*8, *ad*20, *phen*2, *pyro*4, *lys*5 and *s*3 growth requirement, respectively, for biotin, methionine, riboflavin, nicotinic acid, adenine, phenylalanine, pyridoxine, lysine, sulphite; *su*1-*ad*20, suppressor of *ad*20; *Acr*1 (semidominant), *acr*2 and *mg*1 (recessive), non-allelic mutants conferring resistance to acriflavine.

Inhibition by acridines. Two sets of conditions were used. In one, conidia were treated in buffered saline before plating on CM. Dilution on plating was always such as to reduce acridine in the medium to negligible concentration. In the other method, conidia were plated direct on CM (or appropriately supplemented MM) containing acridine. The effect of treatment was measured by ability of conidia to yield a colony after three days' incubation. At least 200 conidia were plated for any one estimation of survival; counts were compared with controls treated identically except for the absence of acridine. During manipulations there were brief exposures to daylight (approx. 100 lumens per square metre).

Photodynamic inactivation. Conidia, in saline at pH 7, were pretreated with acridine, in the complete absence of light, for 2 hours at 25°. They were then illuminated with a tungsten-filament lamp (5 cm. water filter) which gave 8,000 lumens per square metre at the level of the suspension. O_2 or N_2 was bubbled through the suspension during illumination. Treated conidia, and appropriate controls without acridine, were plated on CM and colonies counted after three days.

Fluorescent staining. A system based on that of Clutterbuck & Roper (1965) was used.

Production of morphological variants. Conidia were harvested into saline from 3–5 days old CM cultures, centrifuged, and then suspended in liquid MM supplemented with all growth requirements and with acriflavine at a final concentration of 10 or 40 mg./l. Conidial density was about 3×10^5 per ml. After acriflavine treatment for 24 hours at 37°, saline dilutions were made and the conidia plated on CM so as to yield not more than twenty colonies per dish. No exact control is possible since without acriflavine growth would be substantial after 24 hours in supplemented MM. Two comparisons were used as approximate controls. One involved identical treatment, but without acriflavine, for 4 hours only. In the other comparison, saline was sutsbituted for supplemented MM and treatment, without acridine, was for 24 hours. For comparison experiments aliquots of conidia were taken from a single original suspension. There were brief exposures to normal laboratory lighting during manipulations. Colonies were scored at 3–4 days.

Dark mutation. Cultures of *bi*1; *meth*1 or *bi*1; *Acr*1; *meth*1, purified on each occasion by single colony isolation, were grown on CM for three days at 37°. Conidia were harvested into saline, washed in saline three times and divided for treatment and controls. Treatment was in saline, buffered with phosphate at pH7, and with a final conidial density between 2×10^5 and 2×10^6 per ml. Treatment was for 1 hour at 37° with acriflavine at a concentration of 1 g./l. and O_2 or N_2 was bubbled through the suspension during treatment. Conidia were then washed in saline by centrifugation and plated on screening medium, MM with biotin, to give 2×10^4 to 2×10^5 conidia per dish. Dilutions were plated on CM for viability estimates.

Experiments were carried out in total darkness except for very brief exposure during manipulations to an extremely low intensity yellow light.

Photodynamic mutation. Conidial suspensions were prepared as for dark mutation and then pretreated, for 2 hours at 25°, in the dark, with a non-inhibitory concentration, 10 mg./l., of acriflavine They were then illuminated as for photodynamic inactivation; O_2 or N_2 was bubbled through the suspension during illumination. Controls were as follows: an illuminated suspension which had not been acridine pretreated and suspension, with and without pretreatment,which were not illuminated. Platings were made as for dark mutation.

Reversions were scored at intervals up to six days.

3. RESULTS

(i) *Inhibition*

Strains of *A. nidulans* with genetically determined resistance to the growth inhibitory effects of acriflavine have already been described and certain of the environmental factors affecting the degree of resistance of a strain elucidated (Roper & Käfer, 1957; Warr & Roper, 1965). Preliminary results in the present studies indicated that temperature, pH, stage of life cycle treated and particular acridine used were also relevant to the degree of inhibition. In addition, unexpected interactions were found when pairs of different acridines were used simultaneously. Attention has been concentrated, for the present, on investigating certain of those environmental and genotypic variables which might enhance or protect against any possible acridine-induced mutation.

Two contrasting conditions of treatment were examined. In one, conidia were plated on media containing acridine so that treatment was prolonged during growth. In the other, more suitable for quantitative mutation work and for the control of light, pH and temperature, conidia were treated in suspension and plated on acridine-free medium. In the latter case bound acridine may exert an effect after plating but the widely different results obtained by the two approaches justify their somewhat arbitrary separation.

(*a*) *Inhibition under conditions of growth*

Conidia of a number of strains were plated on supplemented MM with individual acridines. LD_{50} values, derived from the survival curves, are given in Table 1. *Acr*1, *mg*1 and *acr*2 confer resistance, and to the same extent, to AY. Only *Acr*1 conferes resistance to P and all four strains are uniformly sensitive to the remaining acridines.

*Acr*1 is known to be semi-dominant towards acriflavine inhibition (Roper & Käfer, 1957). The only other dominance relationships so far tested were for *mg*1 and *acr*2 towards A and AY. Prototrophic diploids were tested on MM with AY or A. In respect of alleles for resistance their genotypes were $+;+/+;+$, $+/acr2$, $+/mg1$ and $+;+/acr2; mg1$. All four diploids had LD_{50}, on A, of 1·5 mg./l. On

Table 1. *Conidial LD_{50} values for various strains tested against a range of acridines*

Acridine	Strain and LD_{50} * *bi* 1	*bi*1; *mg*1	*bi*1; *acr*2	*bi*1; *Acr*1
acriflavine	1·5	2·5	2·5	24
acridine yellow	14	18·5	18·5	18·5
acridine HCl	70	70	70	70
proflavine	140	140	140	375
5-amino acridine	300	300	300	300
acridine orange †				
atebrin †	1000	1000	1000	1000
coriphosphine †				

* Expressed as mg./l. MM + biotin.
† Approximate values.

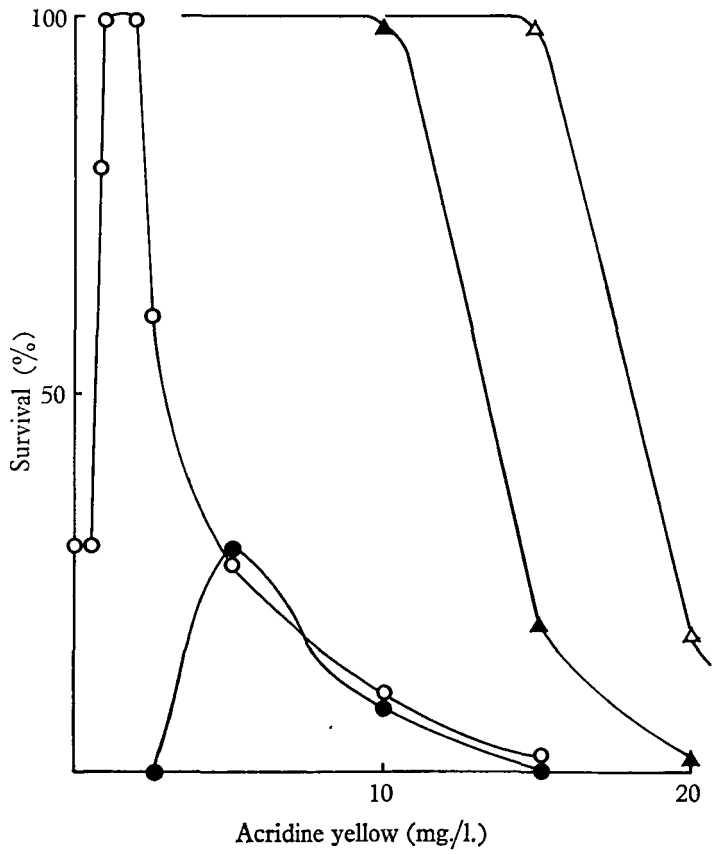

Fig. 1. Interaction of acriflavine and acridine yellow. △, *bi*1; *Acr*1 with AY only; ▲, *bi*1 with AY only; ○, *bi*1; *Acr*1 with 25 mg./l. A; ●, *bi*1 with 25 mg./l. A.

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AY the respective values were (mg./l.), 14, 14, 18·5, 18·5. Thus *acr*2 and *mg*1 are recessive with respect to A. But, with respect to AY, *acr*2 is recessive and *mg*1 dominant.

In a further series of similar experiments pairs of acridines were incorporated into the supplemented MM. All types of interaction were found. Inhibition by AY was not influenced by the simultaneous use of 5A, AT or AO. On the other hand, AC showed potentiation with A and with AY. Conversely, inhibition by A was annulled by appropriate concentrations of AT, AC or AY. Figure 1 shows some of the results and illustrates several features of the interaction of A and AY. First, with a constant level of 25 mg./l. of A, AY annulled the inhibition of both *bi*1 and *bi*1;*Acr*1 although this concentration of A is nearly twenty times the LD_{50} for *bi*1. Second, the curves for *bi*1 and *bi*1;*Acr*1 coincide at higher AY levels and it is no longer possible to distinguish the two genotypes in terms of survival. Finally, at the higher AY concentrations, not by themselves inhibitory, A and AY are additive in effect on both strains.

(*b*) *Inhibition following treatment of conidia in suspension*

A variety of interacting factors may influence the degree of inhibition resulting from acridine treatment in saline. These include temperature, pH, light intensity, ploidy, genotype and availability of O_2.

Conidia of strain *bi*1 showed 100% survival after dark treatment for 24 hours at 25° in saline (phosphate buffer, pH7) with 10 mg./l. A or AO. However, similar treatment at 37° resulted in 10% and 0·1% survival respectively; compared with direct plating on acridine the order of toxicity of the two acridines was reversed.

Table 2 shows the effect of pH on dark treatment in saline. Inhibition was more effective at pH 10 than at pH 3 or pH 7. The relative inhibition at pH 10 was not correlated with the degree of ionisation of the acridine but was correlated with the

Table 2. *Effect of pH on inhibition by acridines of bi*1; *meth*1

pH* acridine	fluorescence	% viability†	percentage ionization acridine‡
3 acridine orange	green	100	100
3 proflavine	green	100	100
3 acriflavine	green	100	100
3 acridine yellow	green	100	100
7 acridine orange	green	100	99·9
7 proflavine	green	100	99·5
7 acriflavine	green	100	100
7 acridine yellow	green	100	99·8
10 acridine orange	red	0·001	55·7
10 proflavine	red & green	50	38·7
10 acriflavine	green	100	99·0
10 acridine yellow	green	100	16·6

* Buffers: borate, phosphate and citrate for pH10, 7, 3 respectively.

† Treatment in each case was for 5 hr., 25°, 20 mg./l. of acridine in buffered saline.

‡ Calculated from Albert (1960).

degree of red fluorescence shown by the treated conidia. The order of toxicity of the acridines under these conditions at pH 10 was different from the order under conditions of growth.

Conidia of strains *bi*1, *bi*1;*Acr*1, *bi*1;*meth*1 and *bi*1;*Acr*1;*meth*1 were treated in saline with acriflavine and without illumination. At various temperatures, times of treatment and acriflavine concentrations the strains showed equal sensitivity. Under these conditions the *Acr*1 allele is not expressed.

High light intensities applied to sensitized conidia resulted, as expected, in photodynamic inactivation. 10 mg./l. of A or AO in phosphate buffered saline was used as photosensitizer; without illumination this treatment would have given 100% viability. Inactivation was found only when O_2 was used; N_2 totally prevented inactivation during a period of 2 hour illumination. The rate of inactivation was dependent on the acridine used as sensitizer and on the ploidy and genotype of the tested strain. Haploid and diploid strains, which did not carry any alleles determining resistance, were compared using AO as sensitizer. The mean LD_{50} and LD_{90} values for two haploids were 20 and 50 min.; for two diploids these values were 45 and 90 min. It was shown above that the *Acr*1 allele does not confer resistance to acriflavine treatment in saline and without illumination. However, this allele is active in protecting against photodynamic inactivation. The LD_{50} values for *bi*1;*meth*1 and *bi*1; *Acr*1;*meth*1 sensitized with A were 50 and 80 min. respectively. A similar difference was observed for *bi*1 and *bi*1;*Acr*1.

The above observations on inhibition have emphasized the overall complexity of action of acridines. They have shown the expected influences and interaction of light and O_2 and have also shown that variation of temperature and pH may affect both the order of toxicity of various acridines as well as the individual degree of toxicity. The mutant alleles which confer growth resistance to acriflavine are highly selective in their actions towards other acridines and do not even confer resistance to acriflavine under all conditions of treatment. It was of obvious interest to compare resistance to growth inhibiton with resistance to any acridine-induced mutation process. Certain pairs of acridines interact in inhibition and this observation again invites comparison with any such interaction in mutation.

(ii) *Mutation*

(*a*) *The production and analysis of morphological variants*

Table 3 summarizes the results obtained by acridine treatment of conidia of various strains under a variety of conditions. Treatment in supplemented MM yielded a higher frequency of variants than treatment in saline, indicating that the process by which variants arise is probably dependent on metabolism. *Acr*1 or, less likely, a component of the residual genotype, protects almost completely against the effects of acriflavine. Classification of morphological variants is generally arbitrary but the types scored were unequivocally abnormal. Overall there was wide range of morphological types, most with reduced linear growth rate. Only broad classification is given here.

Table 3. *Production of morphological variants by acriflavine*

Strain	Acriflavine concentration and treatment medium*	Total colonies examined	% Survival	Variants		% variants
				Stable	Unstable	
*bi*1; *Acr*1	0 mg./l. saline	91	100	0	0	0
*bi*1; *Acr*1	0 mg./l. MM+	357	100	0	0	0
*bi*1; *Acr*1	10 mg./l. saline	116	12·7	0	2	1·5
*bi*1; *Acr*1	10 mg./l. MM+	76	94	0	0	0
*bi*1; *Acr*1	40 mg./l. MM+	174	6·3	0	4	2
*bi*1	0 mg./l. saline	133	100	0	1	1
*bi*1	0 mg./l. MM+	477	100	0	2	0·5
*bi*1	10 mg./l. saline	150	11·3	0	3	1·5
*bi*1	10 mg./l. MM+	171	13	0	17	10
*bi*1	40 mg./l. MM+	151	8·8	0	29	19
*bi*1	40 mg./1. MM+	159	9·2	0	41	26
*bi*1; *meth*1	0 mg./l. MM+	132	100	0	1	1
*bi*1; *meth*1	10 mg./l. MM+	98	7·4	2	11	13
*ribo*1; *y*; *nic*8	0 mg./l. MM+	99	100	0	0	0
*ribo*1; *y*; *nic*8	40 mg./l. MM+	90	9·1	0	11	12
wild type	0 mg./l. MM+	210	100	0	0	0
wild type	40 mg./l. MM+	186	8·9	0	18	10

* Treatment in saline, with or without acriflavine, and in supplemented MM (MM+) with acriflavine, 24 hr. Treatment in MM+ without acriflavine, 4 hr.

There were only two stable variants in a total of some 1300 colonies from treated conidia; both resulted from gene mutation. 'Compact fluffy' resulted from mutation in two freely recombining genes, both located on chromosome II. 'Red stable' resulted from mutation in a single gene located on chromosome I.

The rest of the variants were unstable and were easily lost on subculture, in favour of normal, unless special care was taken to culture from particular areas of a colony. Within the unstable group there were two distinct classes. Class A, comprising less than 10% of the unstables, had near normal linear growth rate and greatly reduced conidiation. Areas of good conidiation appeared within the colony though not as outgrowing sectors. Subculture from such areas yielded only normal growth. Class B had abnormal morphology, perithecia in some cases, reduced growth rate, and showed frequent sectors of normal growth rate and form. Subculture from the sectors yielded only normal colonies; subculture from the abnormal centre yielded both normal and sectoring colonies in variable proportion.

Analysis was attempted of four unstable variants of class B; three of these yielded no definitive information. Each of these three was combined in heterokaryon with a strain of normal morphology. Platings of conidia from the heterokaryons generally gave only colonies with normal morphology. On one occasion a proportion of unstables, in combination with its parental markers, was recovered. In all, 90 hybrid perithecia were sampled from the three heterokaryons; they produced only normal colonies. Only one of these three unstable types was self-fertile; 50 selfed perithecia gave only normal colonies.

Analysis of the fourth class B variant, 'red unstable', induced in strain *bi*1, was more fruitful. This strain was combined in heterokaryon with the strain *su*1-*ad*20 *y ad*20; *Acr*1; *phen*2; *pyro*4; *lys*5; *s*3; *nic*8; *ribo*2. Ascospores were sampled from 18 perithecia. Seventeen of these, 13 hybrid and 4 selfed green, gave only normal colonies. One perithecium gave: normal yellow, 58; normal green, 53; red unstable green, 20; red unstable yellow 17. Ten unstable colonies, five green and five yellow, were examined further by testing conidia from centres and sectors for all segregating markers. In respect of all markers except *pyro* the centres and sectors of each colony showed a uniform phenotype. The allele ratios for these markers were consistent with a 1:1 ratio, the most extreme being 7:3. However, only one colony centre required pyridoxine, as did the sectors arising from this colony. Of the nine colonies whose centres were pyridoxine independent, three gave sectors some of which required and some of which were independent of pyridoxine. 'Red unstable' is an aneuploid disomic for chromosome IV.

(*b*) *Acridine-induced reversions*

Reversion in the *meth*1 system is determined by mutation at ony one of a number of loci (Lilly, 1965). There are three phenotypic classes of revertants, green, brown and small; each class is probably genotypically heterogeneous. Small colonies are the most difficult to score and are probably underscored here. In the range of conidial densities used in this work it was shown that the Grigg effect (Grigg, 1952) did not operate.

Photodynamic mutation. The results of photodynamic mutation are given in Table 4. The concentration of acriflavine used for sensitization is only 1% of that used in dark mutation; thus controls with acridine, but without light, showed no detectable increase in mutation. N_2, as expected, prevents both inactivation and mutation. *Acr*1 affords some protection against inactivation but does not protect substantially, if at all, against mutation.

Mutation in the absence of light. Results are shown in Table 5. Mutation under these conditions is unaffected by O_2, N_2 or the allele *Acr*1. Only one experiment has been undertaken in total darkness throughout because of the difficulty of manipulation under such conditions. Treatment was with 10 mg./l. A for 24 hours at 37°. The lower acriflavine concentration eliminated the need for washing after treatment. In this experiment conidia were embedded in the screening medium. Scoring of both brown and small colonies was difficult but the green class presented no difficulties. Survival following treatment was 10%; equal numbers of green revertants, thirteen in each case, were obtained from equal total numbers of treated and control conidia.

Six revertants, two green, two brown and two small were picked from a treated series. Each was crossed to a *meth*$^+$ strain. All six crosses gave a segregation of methionine independent: methionine requiring which did not differ significantly from the 3:1 ratio expected for mutation at a freely recombining suppressor locus. Only one suppressor, a green, has so far been allocated to a linkage group (VIII) and this adds a further suppressor locus to those located by Lilly (1965).

Table 4. *Photodynamic mutation*

Experiment	Strain	Treatment: Acriflavine	Treatment: Illumination (min.)	Treatment: O_2/N_2	Total conidia screened ($\times 10^5$)	Viability (%)	Revertants: Green	Revertants: Brown	Revertants: Small	Revertants per 10^5 survivors
A	*bi*1; *meth*1	−	0	N_2	20	100	1	17	0	0·9
A	*bi*1; *meth*1	−	0	O_2	20	100	4	10	0	1·0
A	*bi*1; *meth*1	−	60	N_2	20	100	2	12	0	0·7
A	*bi*1; *meth*1	−	60	O_2	20	100	6	18	0	1·2
A	*bi*1; *meth*1	+	0	N_2	20	100	2	11	0	0·7
A	*bi*1; *meth*1	+	0	O_2	20	100	5	19	0	1·2
A	*bi*1; *meth*1	+	60	N_2	20	100	4	18	1	1·2
A	*bi*1; *meth*1	+	60	O_2	20	26	26	18	1	8·7
B	*bi*1; *meth*1	−	0	O_2	5	100	3	8	2	2·6
B	*bi*1; *meth*1	+	60	O_2	5	34	25	23	1	29
B	*bi*1; *meth*1	+	90	O_2	5	15	12	11	0	31
C	*bi*1; *meth*1	−	0	O_2	5·5	100	13	5	0	3·3
C	*bi*1; *meth*1	+	45	O_2	5·5	58	39	15	2	18
C	*bi*1; *meth*1	+	60	O_2	5·5	38	25	12	0	18
D	*bi*1; *Acr*1; *meth*1	−	0	O_2	4	100	3	2	1	1·5
D	*bi*1; *Acr*1; *meth*1	+	60	O_2	4	63	18	13	2	13
D	*bi*1; *Acr*1; *meth*1	+	90	O_2	4	48	29	28	3	31
E	*bi*1; *Acr*1; *meth*1	−	0	O_2	2·9	100	2	2	0	1·4
E	*bi*1; *Acr*1; *meth*1	+	45	O_2	2·9	91	14	15	0	11
E	*bi*1; *Acr*1; *meth*1	+	60	O_2	2·9	80	3	9	0	9·5

The conidia of any one experiment were aliquots from a single suspension

Table 5. *Dark mutation*

Experiment	Strain	Treatment		Total conidia screened ($\times 10^5$)	Viability (%)	Reversions			Reversions per 10^5 survivors
		Acriflavine	O_2/N_2			Green	Brown	Small	
A	*bi*1; *meth*1	−	N_2	20	100	2	1	2	0·3
A	*bi*1; *meth*1	−	O_2	20	100	2	1	0	0·2
A	*bi*1; *meth*1	+	N_2	20	47	25	16	2	4·6
A	*bi*1; *meth*1	+	O_2	20	33	32	8	0	6·1
B	*bi*1; *meth*1	−	O_2	24	100	12	5	2	0·8
B	*bi*1; *meth*1	+	O_2	24	24	24	5	0	5·0
C	*bi*1; *Acr*1; *meth*1	−	O_2	22	100	11	24	0	1·6
C	*bi*1; *Acr*1; *meth*1	+	O_2	22	26	32	19	0	7·2

The conidia of any one experiment were aliquots from a single suspension

One of the revertants, a small, gave considerable vegetative variation in that conidia plated on MM with biotin showed an enormous range of colony sizes. This variation was not shown on CM. From a cross to *meth*$^+$ a quarter of the progeny showed this phenomenon.

4. DISCUSSION

The effectiveness of any one acridine in growth inhibition and killing involves interaction of genotype with conditions of treatment. Overall complexity of action is not surprising. Acridines show affinity for nucleic acids (McIlwain, 1941; Lerman, 1963), proteins (Hass, 1944; Speck & Evans, 1945; Hellerman, Lindsay & Bovarnick, 1946; Sevag & Gots, 1948*a*, *b*) and even, under certain conditions, polysaccharides (Dutta, 1965). Furthermore, Robbins & Marcus (1963) showed that a variety of interdependent factors determine both intracellular distribution of acridine orange and lethality in HeLa cells. Acridines offer scope for indirect effects as well as effects dependent on their complexing with nucleic acids. It may be that in different organisms, and under different conditions, any one of several classes of macromolecules provides the critical site of action.

The present studies on inhibition were undertaken mainly to provide some foundation for mutation work but a number of points relevant to their more general actions have emerged. The influence of temperature and pH on toxicity of any individual acridine and on the relative order of toxicity of a number of acridines may well be related to the findings of Robbins & Marcus (1963) on intracellular distribution. This is supported by the observation that lethality in *Aspergillus* is correlated with the degree of cytoplasmic reddening.

Treatment with pairs of acridines has provided unexpected results which include additivity, potentiation and annulment by one acridine of inhibition by another. Annulment was studied closely only for combinations of acriflavine and acridine yellow. This effect might, as with metabolite–antimetabolite, reflect differential affinity and differential toxicity for the same target. If this is so—and more subtle explanations are by no means excluded—it would indicate that toxicity is not directly related to affinity. The idea of a common target might be tested by a study of photodynamic inactivation, fluorescence and mutation with appropriate pairs of acridines.

One approach in a study of the action of inhibitors is through gene-controlled resistance to them. Some resistance patterns have been determined for strains carrying *Acr*1, *acr*2 or *mg*1. These three non-allelic mutants confer growth resistance to acriflavine; the first, a semi-dominant, determines high resistance while the last two, both recessive, determine a lower degree of resistance. Neder (personal communication) has recently shown that *Acr*1 is temperature sensitive; it is not expressed at 44°. The alleles are highly selective in their actions towards a range of acridines although they all confer resistance to compounds as diverse as acriflavine and certain triphenylmethane dyes (Roper & Käfer, 1957; Warr & Roper, 1965). Moreover, one of these alleles shows different dominance relationships towards

different acridines. The allele *Acr*1 is unexpressed during acriflavine treatment in saline, and acridine yellow annuls acriflavine inhibition of *Acr*1 strains. These two observations suggest that resistance determined by *Acr*1, to both acriflavine and acridine yellow, is an active metabolic process rather than, for example, a matter of simple permeability. However, *Acr*1 does afford substantial protection against acriflavine-sensitized photodynamic inactivation in saline suspension. These observations on inhibition by acridines, and genically determined resistance to it, have done little to elucidate the actions of acridines but they did suggest factors worthy of investigation in any mutation process.

Acriflavine treatment of conidial suspensions, followed by plating on acridine-free medium, can yield over 20% morphological variants among the survivors. The process by which these variants are induced appears to be dependent on metabolism. The allele *Acr*1 affords substantial protection against these acriflavine-induced effects and this is in accord with the suggestions that the induction process and *Acr*1 action are linked to metabolism.

Almost all of the variants are unstable and revert to normal on subcluture unless particular steps are taken to retain them as unstable variants. Analysis of the unstable variants is laborious and was attempted for only four. Three were 'lost' in favour of their normal, stable forms during analysis. The fourth was shown to be an aneuploid, disomic for chromosome IV. Käfer (1961) has considered the question of distinguishing aneuploids from other unstable variants of *Aspergillus* and it is highly relevant here. The red unstable aneuploid had certain of the attributes expected of variants involving an extra-chromosomal determinant; it showed vegetative segregation and was rarely recovered following meiosis of selfed or hybrid zygotes. But for analysis of a cross involving markers on all chromosomes this variant might have been classified tentatively as extra-chromosomal in origin. The aneuploid is in many respects pheno-typically representative of a substantial proportion of the acriflavin-induced variants. We do not, of course, conclude that all members of this phenotypic class are aneuploid; certain tests, such as suppressive 'transfer' in heterokaryons, could definitively exclude aneuploidy in some cases. In the absence of such tests the aneuploid class presents a hazard when acriflavine-induced unstable variants of possible extra-chromosomal origin are being considered.

Certain acridines eliminate the bacterial sex factor (Hirota & Iijima, 1957). Stouthamer, De Haan & Bulten (1963) have shown that this occurs through interference with replication of *F*-particles rather than with their distribution at division. It may be that aneuploids arise through a disturbance of chromosome replication rather than chromosome distribution and that *Acr*1 affords protection against this disturbance.

Photodynamic mutation is a well established phenomenon in other organisms though its results are not yet defined at the level of DNA. Ritchie (1964) suggests that such mutations in phage, with acridines as photosensitizer, may result from changes of the base-analogue type and in *Neurospora* they do not appear to result from base-pair additions and deletions (Brockman, quoted by de Serres, 1964).

The main point of interest in the present study is that *Acr*1, though protecting against photodynamic inactivation, affords no conspicuous protection against photodynamic mutation.

There is evidence to suggest that mutation in the absence of light may, in some systems, require meiosis or other recombination process. There is no significant mutagenic effect of acridines on *Escherichia coli* during vegetative growth (Lerman, 1963); in vegetative cells of *Neurospora* acridines have given negative (Brockman quoted by de Serres, 1964) or equivocal results (Reissig, 1964) while in vegetative growth of yeast 5-amino acridine even has an antimutagenic effect (Magni, von Borstel & Sora, 1964). A similar antimutagenic effect has been observed in bacteria (Webb & Kubitschek, 1963), though Eisenstark & Rosner (1964) did find acridine-induced mutation at certain loci in *Salmonella*. On the other hand certain acridines are mutagenic for vegetative phage (De Mars, 1953) and at meiosis in yeast (Magni, von Borstel & Sora, 1964). The results of Drake (1964) do not permit definitive conclusions on the role of recombination in proflavine-induced mutation of phage; mutation was found in the absence of replication, but mutants did appear to arise in recombinational heterozygotes. Brenner, Barnett, Crick & Orgel (1961) have suggested that acridine mutants are of the base-pair deletion or addition type and Lerman (1963, 1964) has proposed a model to explain the origin of such addition or deletion types by acridine intercalation, between otherwise adjacent DNA base pairs, followed by unequal crossing-over. This model requires recombination as an essential step but encounters a difficulty in that not all those acridines which intercalate with DNA produce mutations in phage.

The present results on 'dark' mutation were obtained with vegetative cells. Manipulation, and therefore accuracy, are difficult in total darkness. Only one experiment has been undertaken under these conditions and on the basis of very small numbers of reversions it suggested a net 10-fold increase in mutation. All other experiments were carried out with very brief exposures to faint yellow light during essential manipulations. Under these conditions acriflavine is a relatively weak mutagen. However, although it is far less effective than β-propiolactone or diepoxybutane applied to the *meth*1 system (Ball & Kilbey, unpublished) it approaches the effectiveness of nitrous acid (Siddiqi, 1962) and it is difficult to suppose that the fairly considerable increase in mutation is due to photodynamic effects alone. Furthermore, mutations occurred with either N_2 or O_2. Six revertants obtained under these conditions have been analysed and each resulted from mutation in a single gene. Our mutation system is not suitable for two-way mutation studies or for fine genetic analysis and, for the present, nothing can be said about the finer details of acridine-induced dark mutations in *Aspergillus*. If our results do indicate dark mutations, and this seems very likely, then recombination as a feature of the process is excluded in the present case. Dulbecco (1964) has suggested that acridines might act as mutagens through the inhibition of error correction. It was proposed above that aneuploids may arise through replication errors and perhaps the same is true, at a finer structural level, of gene mutations.

Acridines are versatile mutagens capable of inducing genic, chromosomal and

extra-chromosomal changes. These studies indicate that through the control of genotype, conditions of treatment and choice of acridine it may be possible to devise selective situations favouring production of any one class of mutants.

SUMMARY

A number of acridines have been tested for ability to inhibit conidia of strains of *Aspergillus nidulans*. The effectiveness of any one acridine in growth inhibition and killing involves interaction of genotype and conditions of treatment such as temperature, pH, treatment medium and light intensity. Mutant alleles which confer growth resistance to acriflavine are selective in their actions towards other acridines, may differ in their dominance relationships with different acridines and are even selective with regard to the conditions under which they confer acriflavine resistance. Certain pairs of acridines, used simultaneously, show additive effects, potentiation, or annulment by one of inhibition caused by the other.

Some of these findings have been applied in a study of factors affecting acridine-induced mutation in *Aspergillus* conidia. Under conditions which permit metabolism, acriflavine induces a high frequency of unstable morphological variants. One such variant has been shown to be a disomic. Using a system of reversion from auxotrophy to prototrophy, acriflavine-induced mutation has been obtained both with high light intensities and in the absence of light. In the latter case recombination as a feature of the mutation process is excluded.

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