COMPETITIVE SUPPRESSION AND THE DETECTION OF MUTATIONS IN MICROBIAL POPULATIONS

By G. W. Grigg*

[*Manuscript received August* 1, 1957]

Summary

In *Neurospora*, high concentrations of auxotrophic cells suppress the growth of prototrophic ones under conditions commonly employed in back-mutation experiments. This phenomenon is discussed in relation to the quantitative detection of rare variants in populations of microbial cells.

The mechanism of the suppression effect is due to the removal of sugar from the medium by non-growing auxotrophic conidia. If enough auxotrophs are present, insufficient free sugar is left for growth of prototrophic conidia to macroscopic colony size.

Other factors which may bias back-mutation experiments are interactions between prototrophic and auxotrophic nuclei within multinucleate conidia and the proportion of dead cells in the conidial population plated. The latter is usually higher on treated than control plates. Data are presented to show that both of these factors operate to enhance the germination and growth rate of prototrophs in treated mixed populations, but not in untreated ones. They may act synergistically with, or independently of, the competitive suppression effect in biasing estimates of the proportion of rare mutants in a microbial population.

I. Introduction

In recent years microorganisms have replaced *Drosophila* as the most commonly used organism in mutation studies. This dates from the time when suitable techniques were developed to select certain types of mutant cells from a population. Such selection methods were developed for the bacteria *Staphylococcus aureus* and *Escherichia coli* by Demerec and his co-workers (Demerec 1946; Demerec and Latarjet 1946; Witkin 1947) when mutants resistant to certain antibiotics or phage were selected by treating sensitive cells with the appropriate agent. By the use of this assay method a large number of substances, including many inorganic salts, were classed as mutagens (Demerec, Bertani, and Flint 1951).

The observation that biochemical mutants of *Neurospora crassa* sometimes acquired the ability to grow on a minimal medium was the basis of an elegant mutation assay method developed independently by Westergaard and co-workers (see Jensen *et al.* 1951) and by Giles and Lederberg (1948). Briefly this method consists of treating a washed suspension of auxotrophic cells with a mutagen, washing further if necessary, and plating on a minimal medium in which only prototrophs will grow. Equal numbers of untreated conidia are plated on a similar medium as controls. Following incubation the numbers of prototrophs are counted on the control and "treated" plates. Any significant differences are ascribed to the mutagenic activity

*Animal Genetics Section, C.S.I.R.O., University of Sydney; present address: Genetics Department, University of Adelaide.

of the treatment. The general assumption is made that any conidium, whether uninucleate (microconidium) or multinucleate (macroconidium), which contains a prototrophic nucleus, will produce a visible colony when plated together with a large number of auxotrophic cells on minimal medium. This will only hold if (i) auxotrophic cells do not inhibit any prototrophs which may be present; (ii) any cell having a nucleus in which back mutation has occurred will immediately germinate and grow as rapidly on minimal medium as on medium supplemented with the growth factor required by the auxotroph.

Inhibition of protrophs by auxotrophs has been shown to occur in *Esch. coli* by Ryan and Schneider (1949) in studies of reversion of a histidine-dependent strain to histidine independence and in *N. crassa* by Grigg (1952) and Stephens and Mylroie (1953). Such inhibition may be demonstrated by plating a prototrophic and an auxotrophic strain, mixed in given proportions, on minimal medium.

In this paper data on which a previous brief report was based (Grigg 1952) will be presented, together with a suggested mechanism for the suppression. The evidence for the widespread nature of the suppression phenomenon in microbial species will be reviewed and its significance discussed.

It will also be shown how the second assumption may be tested with multinucleate conidia and some data given which suggests that the assumption may not always be valid.

II. Material and Methods

The following strains of *N. crassa* were used in these experiments:

Prototrophs

MACROCONIDIAL: 70007 (*col–4**); CMS (*col–1**, *m su*^{m} (Grigg, unpublished data)).

MICROCONIDIAL: CM (*col–1*, *m**).

Auxotrophs

MACROCONIDIAL: W40 (*ad–3*, *col–4** (Kølmark and Westergaard 1949)); 37401 (*inos*); K26·9 (*hist* (Mathieson and Catcheside 1955)).

MICROCONIDIAL: 9a91 (*ad* (accumulates purple pigment), *col–1*, *m*); K51 (*meth*, *col–1*, *m*); K42 (*leuc*, *col–1*, *m*); K26 (*hist*, *col–1*, *m*); K13·5† (*meth*, *col–1*, *m*); K9† (*aden*, *col–1*, *m*); 39401–55 (*nt*, *col–1*, *m*†).

Microconidia first appear on *col–1*, *m* cultures after incubation at 25°C for 7 days (Barratt and Garnjobst 1949). Conidial production continues for about 5 days, but these conidia are very short-lived. It was our experience that higher numbers of viable conidia were obtained if young cultures were used. Macroconidia, which survive for considerably longer periods, were obtained from 4–8-day-old cultures.

The conidia were suspended in sterile distilled water and the suspensions filtered through cotton wool to remove mycelial fragments. They were then centrifuged and resuspended twice in 10-ml quantities of sterile water to free the suspension from growth factors leached from the culture medium. The density of washed conidia

*Barratt *et al.* (1954). The symbol *m* is equivalent to *pe*^{m} of Barratt *et al.*

†Isolated in the Botany Laboratory, Cambridge University. Linkage relations unknown.

in the stock suspension was determined by direct count in a haemocytometer and subsequently the stock suspension was diluted to the appropriate concentration. When it was necessary to determine the concentration of viable conidia, 1-ml aliquots of the appropriate dilution were plated on a medium which supported their growth.

Before sampling cell suspensions either for subsequent dilution or plating, they were vigorously shaken in 1-oz McCartney bottles. Alternate sucking and blowing from a pasteur pipette was insufficient to disperse the clumps of conidia which formed readily if a suspension were left to stand. This is illustrated in Table 1 which compares the effects of the two methods of agitation on the number of colonies which appear following the plating of equal aliquots of two dilutions series.

TABLE 1

PLATING ERRORS DUE TO INSUFFICIENT AGITATION OF SUSPENSIONS PRIOR TO SAMPLING

Number of colonies per plate after incubation for 5 days

Pretreatment	Dilution			
	10^4	10^5	10^6	10^7
Mild agitation by pipette	$41{\cdot}5 \pm 1{\cdot}9$	$12{\cdot}5 \pm 2{\cdot}4$	5·3	1·0
Vigorous mechanical agitation*	$132{\cdot}4 \pm 5{\cdot}0$	$12{\cdot}3 \pm 1{\cdot}2$		

*A sample of different cell concentration from that used in the other treatment group.

Reconstruction experiments.—One-ml aliquots of a dilute suspension of prototrophic conidia containing about 35 viable cells were added to tubes containing 10 ml of melted minimal agar at 40°C and also to tubes of similar medium to which 1 ml of a dense suspension of auxotropic conidia had been added. The aliquots of melted agar medium plus conidia were then poured rapidly into petri plates and incubated at 25°C in a humidified incubator. Usually there were four but sometimes three or five replicates in each group. Colony counts were made 3, 4, and 5 days after plating.

The reconstruction experiments were made on Westergaard and Mitchell's (1947) minimal medium but at pH 5·6 instead of pH 6·5 and containing either 2 per cent. glucose or 0·2 per cent. sucrose plus 0·4 per cent. sorbose as the carbohydrate source. The complete medium used was the conidiating medium of Horowitz (1947).

III. RESULTS

(*a*) *Reconstruction Experiments*

All of the auxotrophic strains tested, including the strains W40 and 37401 which have been used extensively in mutation experiments (Giles and Lederberg 1948; Dickey, Cleland, and Lotz 1949; Jensen, Kølmark, and Westergaard 1949; Kølmark and Westergaard 1949; Jensen *et al.* 1951; Smith and Srb 1951), inhibited prototrophs.

TABLE 2

SUMMARY OF RESULTS OF RECONSTRUCTION EXPERIMENTS

Auxotrophic Strain		Prototrophic Strain	Medium*	No. of + Colonies per Plate following Plating of:		No. of Viable Auxotrophic Conidia per Plate ($\times 10^5$)
				+Conidia	+and Auxotrophic Conidia	
Macroconidial strains	W40	CM	2% glucose	28·6±3·0	38·6±3·5†	230
				46·5±4·4	0·3	2700
	W40	CMS	2% glucose liquid	Heavy growth	No growth	150
	W40	CMS	Sorbose-sucrose	52·0±3·2	1·7	31
	W40	CMS	Sorbose-sucrose	52·0±3·2	10·3±3·0	3·1
	W40	70007	Sorbose-sucrose	127·5±6·9	138·8±10·7†	320
				27·0±3·0	22·0±0·9†	250
				167·0±9·8	0	380
	37401	CM	Sorbose-sucrose	160·0±3·9	0	820
	37401	70007	Sorbose-sucrose	54·8±3·6	4·3±3·6	400
	K26·9	CM	Conidiating, complete	150·5±5·7	5·7±0·7	200
			2% glucose	500	205±16·5	170
	K26·9	CM	Sorbose-sucrose	20·2±2·3	0	20
Microconidial strains	9a91	CM	Sorbose-sucrose	55·0±6·6	6·5±0·5	37·5
	39401–55	CM	Sorbose-sucrose	191·0±27·3	0	930
				191·0±27·3	49·3±1·8	930
	K51	CM	Sorbose-sucrose	60·0±2·5	1·5	1100
	K9	CM	Sorbose-sucrose	60·0±2·5	15·0±2·1	370
				36·0±3·3	1·5	600
	K42	CM	Sorbose-sucrose	12·5±2·5	0	1500
	K26	CM	Sorbose-sucrose	11·0±1·9	3·8±1·1	29
				40·3±2·3	0	320
	K13·5	CM	Sorbose-sucrose	16·2±2·2	0	108

*Agar medium except where indicated. †Size of colonies very much smaller than on control plates—see Plate 1, Figure 1.

Many of the experiments were performed on the low carbohydrate sorbose-sucrose medium but qualitatively similar results were obtained when 2 per cent. glucose minimal medium was substituted. The concentration of auxotrophs necessary to suppress prototrophs was much higher on the glucose than on the sorbose-sucrose medium. For example, 3×10^6 microconidia from a W40 adenineless culture were sufficient to suppress prototrophic microconidia completely when added to 10 ml of sorbose-sucrose medium, but 3×10^7 conidia were necessary when glucose medium was used.

It is apparent that macroconidia from auxotrophic cultures have a greater suppressing capacity than microconidia. 9a91 and W40 carry the adenine-purple gene, but 10 times the number of microconidia from 9a91 are necessary to suppress prototrophic conidia as macroconidia from W40. Also a higher number of auxotrophic conidia are needed to suppress macroconidia from the prototrophic strain 70007 than microconidia from CM.

It is of interest that a similar number of *ad*-purple microconidia from 9a91 were required to suppress prototrophic microconidia as *ad*-purple macroconidia from W40 to suppress prototrophic macroconidia 70007. A subinhibitory dose of W40 macroconidia did not reduce the number of prototrophic colonies produced but did cause a considerable reduction in their size compared with the controls (Table 2; Plate 1, Fig. 1).

Since the medium containing 2 per cent. glucose is the one most used in back-mutation experiments in *Neurospora* the results obtained with it deserve special attention. Complete suppression of prototrophic microconidia occurred when they were mixed with 3×10^7 W40 macroconidia and plated in 10 ml of glucose medium (Table 2). When reconstruction experiments were performed with macroconidia of W40 and of Kølmark and Westergaard's (1953) *circularis* strain, inhibition of the prototrophs, as shown by a decrease in colony diameter compared with the controls, was caused by $1{\cdot}9 \times 10^7$ W40 conidia per plate. A five-fold decrease in colony size was induced by $3{\cdot}7 \times 10^7$ W40 conidia. Many of the colonies were barely visible. Suppression of prototrophic macroconidia by conidia from W40 was observed in liquid medium containing 2 per cent. glucose. Thus despite Kølmark and Westergaard's (1952) claim to the contrary some inhibition of prototrophic cells by W40 conidia seems to occur at concentrations commonly used in mutation experiments.

Relatively low numbers of macroconidia ($1{\cdot}7 \times 10^6$) per 10 ml of medium, from a histidineless strain (K26·9) caused a considerable inhibition of prototrophs on glucose medium. Both the number of colonies and their size were considerably diminished. A concentration of 2×10^7 K26·9 conidia was sufficient to produce complete suppression of macroconidial prototrophs when plated on a high carbohydrate conidiating medium containing 2 per cent. glycerol (Horowitz 1947).

These observations suggested that the apparent mutagenic activity of some treatments such as radiations might be explicable on the basis of their known lethal effects if the inhibition of prototrophs caused by the dead cells was smaller than that caused by living cells. Consequently several reconstruction experiments were performed in which dead W40 conidia, killed by ultraviolet irradiation, were mixed with prototrophic microconidia and plated on minimal medium. The numbers of

prototrophic colonies on the minimal and minimal plus dead conidia medium did not differ, nor were the prototrophic colonies reduced in size, even when as many as 5×10^7 dead conidia were added to each plate; on the contrary, the prototrophic colonies appeared much sooner after plating of conidia on the "minimal plus dead conidia" medium than on the minimal medium (Table 3). This acceleration of growth of prototrophs on medium containing dead conidia is of importance when considering the reliability of platings of untreated viable auxotrophic cells as controls of mutation experiments.

(*b*) *Dilution Experiments*

Because wild-type conidia are inhibited by auxotrophic conidia, any prototrophic ones present as a result of spontaneous mutation in a population of auxotrophic cells should not grow on a plate containing minimal medium if an inhibiting concentration of auxotrophic conidia is present. If wild-type cells were in the mutant population then dilution of a cell suspension to a level less than the inhibitory one might allow wild-type colonies to appear.

TABLE 3

EFFECT OF DEAD W40 CONIDIA IN THE MEDIUM ON THE GERMINATION RATE OF PROTOTROPHIC CONIDIA

Number of prototrophic colonies per plate visible 3 days after the addition of equal numbers of prototrophic conidia to minimal medium and minimal medium plus dead cells

Experiment	Minimal Medium	Minimal Medium plus Dead Cells
1	0	6·3±1·4
2	31·8±6·2	62·7±1·5
3	5·8±0·9	14·0±1·5
4	0	5·5±1·3

From the results obtained dilution experiments varied considerably from strain to strain. In some, e.g. K42, the yields of prototrophs were greater at low concentrations than would be expected from the number which appeared at higher concentrations, in others, e.g. W40, a decrease in the number of conidia per plate resulted in a proportionate decrease in the number of prototrophic colonies detected (cf. Kølmark and Giles 1955). Variable results were obtained with other strains. Qualitatively similar data were observed whether the dilutions were performed on sorbose-sucrose or glucose medium. The data from one of the series of dilution experiments are shown in Table 4(*a*). The occurrence of prototrophs in this irradiated population of K42 cells can be adequately explained on the measured lethal effects of the radiation without assuming that the treatment was mutagenic (Table 4(*b*) and Plate 1, Fig. 2). Similar results were obtained when serial dilutions of conidia from some but not all 9a91 cultures were plated. The discrepancy may have arisen as a result of differences in the small proportion of macroconidia with their greater suppressing ability (10–50 times that of microconidia) in the otherwise microconidial population or simply because of a real difference in the proportion of prototrophs present in the populations plated.

Although no regular "dilution effect" was observed with macroconidia from strains W40 and 37401, occasionally larger numbers of prototrophs appeared in diluted samples than were expected from the numbers observed in the more concen-

TABLE 4

DILUTION AND IRRADIATION EXPERIMENTS

(*a*) Number of prototrophic colonies per plate visible 4 days after the plating of several concentrations of conidia from strains 9a91 (*ad*-purple) and K42 (*leuc*)

	Dilution			
	0	10	10^2	10^3
Strain K42				
Mean number of colonies per plate	0·8	22·6±3·5	*c.* 200	17·5±0·81
No. of viable auxotrophic conidia per plate	5×10^7	5×10^6	5×10^5	5×10^4
Strain 9a91				
(i) Mean number of colonies per plate	0	106·3±21·4	3·2±1·9	—
No. of viable auxotrophic conidia per plate	5×10^7	5×10^6	5×10^5	—
(ii) Mean number of colonies per plate	*c.* 1000*	249·0±49·0	22·0±4·0	1·0
No. of viable auxotrophic conidia per plate	$2\cdot1\times10^7$	$2\cdot1\times10^6$	$2\cdot1\times10^5$	$2\cdot1\times10^4$

(*b*) Number of prototrophic colonies appearing after ultraviolet irradiation of K42 conidia compared with those appearing when unirradiated conidial suspensions are diluted and plated

	Unirradiated				Ultraviolet Irradiated	
	Dilution				Dilution	
	0	10	10^2	10^3	0	10
Mean number of colonies per plate	0	45·6±2·4	*c.* 500	42·2±7·9	Large number	91·5±2·5
No. of viable auxotrophic conidia per plate	$2\cdot0\times10^7$	$2\cdot0\times10^6$	$2\cdot0\times10^5$	$2\cdot0\times10^4$	$11\cdot0\times10^5$	$11\cdot0\times10^4$
No. of colonies per 10^6 auxotrophs plated	0	22·8	*c.* 2500	21·10	—	827

*Minute colonies, appeared several days after others.

trated samples. In multinucleate macroconidia there is the possibility that auxotrophic nuclei interfere with the expression of prototrophic ones sharing the same cytoplasm (Grigg 1952). Some evidence of this interference is given below.

TABLE 5

INTRACONIDIAL INTERACTION BETWEEN PROTOTROPHIC AND AUXOTROPHIC COLONIES

Number of colonies per petri plate after incubation of conidia from two histidineless–prototrophic heterokaryons at 25°C

(*a*) Heterokaryon 1* $h^+ : h :: 1 : 2$

	Sorbose Minimal Medium				Sorbose Minimal Medium plus 30 μg/ml Histidine			
	Days of Incubation				Days of Incubation			
	3	4	5	6	3	4	5	6
Total	64	302	383	415	278	463	527	545
Mean	16·0±3·7	75·5±6·2	95·7±5·9	103·7±5·5	69·5±4·3	115·7±6·0	131·7±6·3	136·2±6·6
Mean of estimated number of prototrophic colonies per plate	16·0±3·7			103·7±5·5	50·5±2·8			99·5±4·3
Mean number of "late" colonies	103·7±5·5−16·0±3·7 = 87·7±8·5				99·5±4·3−50·5±2·8 = 49·0±1·8			

(*b*) Heterokaryon 2† $h^+ : h :: 2 : 1$

Total	59	223	283	299	51	131	177	211
Mean	14·7±2·3	55·7±5·5	70·7±2·0	74·7±2·5	17·0±6·7	43·7±3·9	59·0±3·0	70·3±1·2
Mean of estimated number of prototrophic colonies per plate	14·7±2·3			74·7±2·5	17·0±6·7			67·3±1·2
Mean number of "late" colonies	74·7±2·5−14·7±2·3 = 60·0±3·7				67·3±1·2−17·0±6·7 = 50·3±6·8			

*Viability of conidia plated = 80 per cent. †Viability of conidia plated = 60 per cent.

(c) *Intracellular Suppression*

The second assumption of the back-mutation assay procedure, that there is no intracellular suppression in multinucleate cells or phenotypic lag in uninucleate cells, is more difficult to test than the first. Some information can be obtained by studying germination of conidia from heterokaryons of the constitution a–a^+ where a is an auxotrophic mutant. Such studies with artificial heterokaryons have indicated that under certain conditions auxotrophic nuclei interfere with the free expression of the wild-type nuclei.

The metric used was the rate of appearance of macroscopic colonies. This rate was expressed as the number of heterokaryotic colonies visible macroscopically on minimal and on supplemented medium at times t_1, t_2, etc. If the auxotrophic nuclei in the heterokaryotic conidia did not affect the expression of the wild-type nuclei the germination on minimal and supplemented medium should be equal to that of homokaryotic wild-type conidia on the same media.

Aliquots of conidia from two *hist*–*hist*$^+$ heterokaryons differing only in their nuclear ratios were plated on minimal medium and on histidine-supplemented medium. The colonies were labelled as they appeared and the numbers recorded during the following 6 days. The data from the plating experiments are recorded in Table 5.

An estimate of the number of prototrophic colonies (both hetero- and homokaryotic) on the histidine-supplemented plates can be obtained by subtracting from the total on each day the calculated proportion of histidineless colonies, assuming equal germination rates of wild-type and *hist* histidineless conidia on histidine-supplemented medium when tested. Actually the *hist*$^+$ conidia germinated more rapidly than the *hist* ones so this simplification will tend to decrease in magnitude any observed differences between them. The estimated numbers, n_1' and n_2' of prototrophic (hetero- and homokaryotic) colonies per plate after incubation for 3 days are given in Table 5.

In heterokaryons 1 and 2 grown on complete medium, the proportion of heterokaryotic conidia expected on the assumption of random segregation of nuclei into the multinucleate conidia is 68 per cent. (estimated by the method of Prout *et al.* 1953). Seven per cent. in heterokaryon 1 and 25 per cent. in heterokaryon 2 were expected to be wild type. Hence even though it was not possible to distinguish wild type from heterokaryotic colonies in the data the fact that the latter were far more frequent makes it possible to detect a lowered germination rate of the heterokaryons on minimal medium should it exist.

It is apparent from the data in Table 5 that in heterokaryon 1 the number of prototrophic colonies visible on the third day of incubation was much lower on minimal than on histidine-supplemented medium although the final numbers of heterokaryotic colonies visible on the sixth day on both media were equal. The difference is highly significant ($P \leqslant 0{\cdot}01$). On the other hand, no difference was apparent with the second heterokaryon. It will be recalled that this heterokaryon had a higher proportion of wild-type to histidineless nuclei than the other one. This second plating experiment demonstrated that the addition of histidine to the medium did not affect

the germination rate of wild-type conidia or conidia from a heterokaryon having only a low proportion of histidineless nuclei.

These experiments are interpreted to show that if present in the proportion *hist*$^+$: *hist* :: 1 : 2, histidineless nuclei interfere with the free expression of the *hist*$^+$ nuclei, whereas no such effect is demonstrable when *hist*$^+$: *hist* :: 2 : 1.

Similar experiments were performed with an argininelessâ€“tyrosineless heterokaryon, the components of which carried complementary albino markers and additional biochemical mutant genes. The heterokaryon had the wild-type colour (pink). No difference in germination rate was observed between the minimal medium and media supplemented with arginine, tyrosine, or arginine and tyrosine.

TABLE 6

MECHANISM OF SUPPRESSION 1

Colony counts after incubation of plates at 25°C for 5 days, showing the failure of growth of prototrophic conidia on a culture filtrate of K26·9 or W40 conidia in minimal medium, compared with that on a medium consisting of the filtrate plus sugars

	Agar, K26·9 Filtrate*	Agar, Sugars, K26·9 Filtrate*	Agar, W40 Filtrate†	Agar, Sugars, W40 Filtrate†
Total	81‡	139	5‡	156
Mean	$20{\cdot}2 \pm 12{\cdot}4$	$34{\cdot}8 \pm 3{\cdot}9$	$1{\cdot}7 \pm 1{\cdot}2$	$39{\cdot}0 \pm 4{\cdot}4$

*The culture filtrate from a suspension of $2{\cdot}5 \pm 0{\cdot}3 \times 10^7$ K26·9 conidia was added to each petri plate of medium.

†Each petri plate of medium contained the culture filtrate from a suspension of $3{\cdot}95 \times 10^7$ W40 conidia.

‡Size of colonies much less than in other group.

(*d*) *Mechanism of High Density Suppression*

Two general mechanisms by which one cell type may suppress another are (1) the inhibiting species may produce some substance toxic to the other cell species, (2) it may remove some substance from the medium which the other cell type requires for growth. That the culture filtrate from medium in which an inhibiting dose of K26·9 conidia had been incubated for 4 days contained no appreciable content of toxic substance was apparent when its addition to fresh medium produced no obvious inhibition of colony growth.

On the other hand, addition of fresh salts and glucose to a culture filtrate which did not support growth of a prototrophic inoculum resulted in immediate resumption of normal growth. Washed conidia were incubated in 8-ml aliquots of liquid minimal medium for 3 days at 25°C. The filtrate from each culture was heated to 95°C for 15 min prior to mixing with agar (0·2 g) alone or plus sugar. After cooling to 40°C aliquots of prototrophic conidia were added, the mixture poured into petri dishes, and incubated at 25°C. Sugar added alone to culture filtrates of a histidineless K26·9 and an adenineless W40 strain was sufficient to produce normal growth of prototrophic conidia inoculated, as is shown in Table 6.

It has been observed (Table 2) that a concentration of auxotrophic conidia sufficient to suppress prototrophic conidia completely on a low-sugar medium caused no observable suppression on a high-sugar (2 per cent.) medium. Therefore auxotrophic conidia (K26·9) were incubated in media containing a low (0·2 per cent.) and a high (2·0 per cent.) concentration of sugar. Prototrophs added subsequently did not grow to macroscopic size on the low-sugar culture filtrate, but growth was quite normal on the high-sugar culture filtrate medium as it was on low-sugar filtrate plus added sugar medium (Table 7). Presumably the auxotrophs removed most of the sugar from the low-sugar medium, but only a proportion of that in the high-sugar medium leaving sufficient for normal growth of the prototrophs.

TABLE 7

MECHANISM OF SUPPRESSION 2

Growth of prototrophic conidia on a culture filtrate from a suspension of K26·9 conidia in a low-sugar minimal medium (0·2 per cent.) compared with that on a high-sugar minimal medium (2·0 per cent.), sugar-supplemented culture filtrate, and minimal medium. Equal aliquots of prototrophic conidia were added to all plates of medium. Colony counts after incubation of plates at 25°C for 5 days

	Minimal Medium	Agar, K26·9 Filtrate (low sugar)*	Sorbose-sucrose, K26·9 Filtrate (low sugar)*	Agar, K26·9 Filtrate (high sugar)*	Sorbose-sucrose, K26·9 Filtrate (high sugar)*
Total	111	1	194	56	65
Mean	27·8±3·7	0·25±0·25	48·5±7·6	18·7±0·9	21·7±1·5

*Each petri plate of medium contained the culture filtrate from a suspension of $2{\cdot}8 \times 10^7$ K26·9 conidia.

A periodical sampling of a suspension of auxotrophic conidia in liquid minimal medium and a manometric assay of the glucose content of the sample using glucose oxidase verified this presumption. Samples were removed simultaneously from flasks containing only minimal medium and minimal plus auxotrophic conidia. In the absence of inositol, conidia from the inositolless strain 37401 removed $2{\cdot}52 \times 10^{-7}$ mg glucose per day from the medium in a linear fashion for 4 days when uptake ceased abruptly. The uptake from inositol-supplemented medium followed the same curve as the unsupplemented one for the first 2 days, but thereafter increased rapidly.

IV. DISCUSSION

(*a*) *The Suppression Phenomenon*

Of the factors contributing to suppression of prototrophs by auxotrophs perhaps the most important are (i) the removal of sugar by the auxotroph, and (ii) the growth rate of the prototroph.

(i) *Sugar Uptake by Auxotrophs.*—Since the addition of sugar to a culture filtrate from a suspension of non-growing auxotrophic conidia in minimal medium restored the ability of the filtrate to support the growth of prototrophic conidia, plus the fact

that the auxotrophs took up sugar from the medium, it seems likely that removal of sugar from the medium by the auxotrophic cells was involved in the mechanism of the suppression. The sudden drop in sugar uptake of the inositol-requiring strain 37401 after 3 days on minimal medium was probably due to death of most of the conidia. It is of interest that in the first 2 days after inoculation the inositolless conidia removed from the medium the same amount of sugar on minimal as they did on inositol-supplemented medium.

If the sugar level in the minimal medium is reduced below that necessary for growth of the prototrophs before they have reached macroscopic colony size no prototrophs will be detected macroscopically. An increase in the sugar content of the plating medium diminishes the suppresssion effect as does a decrease in the number of inhibiting auxotrophic cells present.

Ryan and Schneider (1949) using *Esch. coli* have shown that under anaerobic conditions the suppression of prototrophs by histidine-requiring bacteria was brought about by a somewhat similar mechanism.

(ii) *Growth Rate of the Prototroph.*—It might be anticipated that the germination and growth rates of the particular prototrophic species present would be an additional important factor. The observation that a greater concentration of auxotrophic conidia must be present to suppress the prototrophic macroconidia than the more slowly germinating microconidia confirms this.

Prototrophs grew more rapidly on medium containing radiation-killed cells than on medium containing none (Table 3). It has been demonstrated that even non-lethal irradiation can stimulate growth (Adelstein *et al.* 1952). Thus prototrophs present in an irradiated cell population might be expected to form macroscopic colonies sooner than prototrophs in a non-irradiated population and hence have a lesser chance of being suppressed by a given number of auxotrophs than the latter.

In back-mutation experiments with macroconidial strains of *Neurospora*, prototrophs are usually in the form of heterokaryons with nuclei of the parental auxotroph. In estimating the importance of intercellular suppression in such a situation by means of reconstruction experiments this fact should be recognized since, as we have seen with histidineless–wild-type heterokaryons, heterokaryotic conidia may grow or germinate more slowly than prototrophic homokaryons on minimal medium. The extent of the intracellular inhibition increased with increase in proportion of histidineless nuclei, within the restricted range of nuclear ratios used. It is not unreasonable to suppose that heterokaryotic conidia from a histidineless strain containing a very low proportion of spontaneous back-mutants would show an even slower growth on minimal medium. Growth might be so slow that such conidia would not form macroscopic colonies before the experiment were concluded and hence would not be scored. Irradiated heterokaryons will have, on the average, a lower number of inhibiting auxotrophic nuclei per conidium and consequently might be expected to have a more rapid germination rate. Again, the small amounts of growth substance released into the medium from killed cells would tend to increase the germination rate of the treated heterokaryotic conidia compared to the non-treated ones.

These various effects all tend to increase the chance of prototrophic nuclei, present before treatment, forming macroscopic colonies on treated plates as compared

with the control plates. Account should be taken of them when performing control reconstruction experiments to test for competitive suppression.

(*b*) *Mutation Assay in* Neurospora

It has been claimed that W40 *circularis* conidia do not inhibit prototrophs on 2 per cent. glucose medium (Kølmark and Westergaard 1952). We found, however, that the maximum concentration of W40 conidia used by these workers caused inhibition of growth of added prototrophic conidia on this medium (shown as a considerable reduction in size of prototrophic colonies compared with the controls). No prototrophic colonies appeared when double this concentration of W40 conidia was used. The suppression of prototrophic conidia by auxotrophic ones at concentrations commonly used in mutation experiments has been confirmed by Stephens and Mylroie (1953).

TABLE 8

BACK-MUTATION RATE OF INOSITOLLESS CONIDIA FOLLOWING IRRADIATION WITH ULTRAVIOLET AND VISIBLE LIGHT

Data extracted from Table 1 of Brown (1951)

Dosage of Ultraviolet (ergs/mm^2)	Number of Survivors Tested ($\times 10^{-6}$)	Reversion Frequency per 10^6 Viable Cells
1500	26	9
	50	4·5
	52	4
2000	13	14
	25	8
	26	3·7
2500–3000	1	135
	2·5	96
	10	25
	13	8·3

Anomalous results in microbial mutation experiments reported in the literature can be explained readily by suppression of rare wild-type cells by the mutant ones. Thus the observation of Brown (1951) that as many prototrophic colonies appeared at 1/100 as at 1/10 dilution when microconidia from an inositol-requiring strain were plated on minimal medium is strongly suggestive of high density suppression. A similar dilution phenomenon was described for some other microconidial auxotrophs by Grigg (1952) and also in this paper. In these the growth of prototrophic conidia derived by spontaneous mutation was suppressed on the plates containing the greatest density of auxotrophs, but as the density of auxotrophs decreased the number of visible prototrophic colonies increased. A comparison of the mutation rates given in Table 1 of Brown (1951) resulting from each treatment with the number of cells plated suggests a relation between the number of survivors tested and the reversion frequency (Table 8). The reversion frequency seems independent of the dosage and dependent

only on the numbers of survivors. When 26×10^6 or more survivors were plated the reversion frequency was moderately low but when fewer than 13×10^6 survivors were plated the "reversion frequency" increased very considerably. Because of the likelihood that competitive suppression was influencing her results Brown's interpretation of them may be queried.

It is apparent that cell interactions of the type discussed here can seriously bias interpretation of data in mutation experiments, but do they invalidate the claims that various chemicals and irradiations increase the back-mutation rate in microorganisms? No general answer can be given to this since most workers have ignored the necessity to test the assumptions inherent in their techniques of recovering "induced" reversions. Of the published work in which an attempt has been made to do this, that of Giles (1951) and Kølmark and Giles (1955) is the most impressive and Giles' interpretation, that various treatments including ionizing radiations increased the back-mutation frequency, therefore, seems reasonable.

Irradiation does not increase the back-mutation rate of all biochemical mutants of *Neurospora* as was illustrated by the observations on the nicotinicless strain 39401; in fact, those on which most of the published work has been done, namely 37401 and W40, are probably selected samples. It may well be that a search will locate numerous loci in *Drosophila* and maize which show a behaviour to irradiation similar to *Neurospora* genes 37401 and W40. The differences in the genetic behaviour to irradiation between microorganisms and higher organisms which have been claimed (Lefevre 1950) may prove fallacious.

(*c*) *Bacterial Plating Experiments*

Competitive suppression seems of widespread occurrence in experiments with bacteria. During the course of an elegant series of experiments on mutation in a histidine-dependent strain of *Esch. coli*, Ryan and Schneider (1949) found that prototrophs did not multiply in the presence of 7×10^9 histidine-dependent cells per 5 mg glucose in the medium. The authors commented that the growth restriction observed may be of general occurrence in mixtures of auxotrophic and prototrophic cells. That a similar phenomenon operates in mixtures of cells sensitive and resistant to drugs and phage is apparent. For instance Saz and Eagle (1953) observed that the addition of penicillin-sensitive bacteria prevented the growth of penicillin-resistant ones in the presence of penicillin. In explanation they postulated a mechanism involving the release of a species-specific toxic factor from the inactivated sensitive cells which killed the resistant ones. It seems more likely that the inactivated cells inhibited growth of the resistant ones, perhaps by removing a nutrient from the medium. Newcombe (1948) reported an apparent decline in the mutation rate of phage-sensitive to phage-resistant *Esch. coli* cells when densities greater than 5×10^9 sensitive cells per plate were used. An increase in the number of back-mutant colonies with increase in concentration of mutant cells plated has been observed in several different microorganisms, e.g. *Esch. coli* (Bryson 1948), *Pseudomonas fluorescens* (Engelsberg 1952), *Brucella abortus* (Olitzki 1952, 1953). Despite the varied explanations put forward by these authors to explain their observations the simplest is that back-mutant cells present in the initial cell population were suppressed when the

concentration of cells plated exceeded a certain threshold. Since this threshold is likely to vary with the medium used it is not valid to carry out controls of the plating technique on one medium and to extrapolate the results to another.

Jinks (1952) has suggested that competitive suppression similar to that described in this paper could bias *Esch. coli* linkage data. Some data of Nelson (1951) in which an apparent increase in prototroph recombinants was associated with a decrease in the concentration of plated auxotrophs could be interpreted to support this view. In spite of these possible misinterpretations, it is unlikely that competitive suppression has been an important source of error in the interpretation of recombination experiments (Ryan 1953).

Competitive suppression seems of widespread occurrence in microorganisms. In consequence, adequate control reconstruction experiments should be part of any experiments where the frequency of one rare cell type is to be determined by plating methods.

V. Acknowledgments

I am greatly indebted to Professor D. G. Catcheside, Dr. M. Jean Mayo, and Dr. J. M. Rendel for helpful discussion and criticism.

VI. References

Adelstein, S. J., Hershey, F. B., Loojbourow, J. R., and Sizer, I. W. (1952).—The stimulation of yeast growth and respiration by compounds produced by yeast cells irradiated with ultra-violet light. *J. Cell. Comp. Physiol.* **40**: 269.

Barratt, R. W., and Garnjobst, L. (1949).—Genetics of a colonial microconidiating mutant strain of *Neurospora crassa*. *Genetics* **34**: 351.

Barratt, R. W., Newmeyer, D., Perkins, D. D., and Garnjobst, L. (1954).—Map construction in *Neurospora crassa*. *Advanc. Genet.* **6**: 1.

Brown, J. S. (1951).—The effect of photoreactivation on mutation frequency in *Neurospora*. *J. Bact.* **62**: 163.

Bryson, V. (1948).—Effects of nitrogen mustard on *Escherichia coli*. *J. Bact.* **56**: 423.

Demerec, M. (1946).—Induced mutations and possible mechanisms of the transmission of heredity in *Escherichia coli*. *Proc. Nat. Acad. Sci., Wash.* **32**: 36.

Demerec, M., Bertani, G., and Flint, J. (1951).—A survey of chemicals for mutagenic action on *Escherichia coli*. *Amer. Nat.* **85**: 119.

Demerec, M., and Latarjet, R. (1946).—Mutations in bacteria induced by radiations. *Cold Spr. Harb. Sym. Quant. Biol.* **11**: 39.

Dickey, F. H., Cleland, G. H., and Lotz. C. (1949).—The role of organic peroxides in the induction of mutations. *Proc. Nat. Acad. Sci., Wash.* **35**: 581.

Engelsberg, E. (1952).—The mutagenic action of formaldehyde on bacteria. *J. Bact.* **63**: 1.

Giles, N. H. (1951). Studies on the mechanism of reversion in biochemical mutants of *Neurospora crassa*. *Cold Spr. Harb. Sym. Quant. Biol.* **16**: 283.

Giles, N. H., and Lederberg, E. Z. (1948).—Induced reversions of biochemical mutations in *Neurospora crassa*. *Amer. J. Bot.* **35**: 150.

Grigg, G. W. (1952).—Back-mutation assay method in microorganisms. *Nature* **169**: 98.

Horowitz, N. H. (1947).—Methionine synthesis in *Neurospora*: the isolation of cystathionine. *J. Biol. Chem.* **171**: 255.

Jensen, K. A., Kirk, I., Kølmark, G., and Westergaard, M. (1951).—Chemically induced mutation in *Neurospora*. *Cold Spr. Harb. Sym. Quant. Biol.* **16**: 245.

JENSEN, K. A., KØLMARK, G., and WESTERGAARD, M. (1949).—Back mutation in *Neurospora crassa* induced by diazomethane. *Hereditas* **35**: 521.

JINKS, J. L. (1952).—Competitive suppression and the determination of linkage in micro-organisms. *Nature* **170**: 106.

KØLMARK, G., and GILES, N. H. (1955).—Comparative studies of monoepoxides as inducers of reverse mutations in *Neurospora*. *Genetics* **40**: 890.

KØLMARK, G., and WESTERGAARD, M. (1949).—Induced back mutations in a specific gene of *Neurospora*. *Hereditas* **35**: 490.

KØLMARK, G., and WESTERGAARD, M. (1952).—Validity of the *Neurospora* back-mutation test. *Nature* **169**: 626.

KØLMARK, G., and WESTERGAARD, M. (1953).—Further studies on chemically induced reversions at the adenine locus of *Neurospora*. *Hereditas* **39**: 209.

LEFEVRE, G. (1950).—X-ray induced genetic effects in germinal and somatic tissue of *Drosophila melanogaster*. *Amer. Nat.* **84**: 341.

MATHIESON, M. J., and CATCHESIDE, D. G. C. (1955).—Inhibition of histidine uptake in *Neurospora crassa*. *J. Gen. Microbiol.* **13**: 72.

NELSON, T. C. (1951).—Kinetics of recombination in *Escherichia coli*. *Genetics* **36**: 162.

NEWCOMBE, H. B. (1948).—Delayed expression of mutations. *Genetics* **33**: 447.

OLITZKI, A. L. (1952).—The determination of the most probable numbers of streptomycin-fast cells in *Brucella* cultures and their variability in growing and aging cultures. *J. Gen. Microbiol.* **6**: 166.

OLITZKI, A. L. (1953).—Growth inhibition of streptomycin-resistant mutants in dense suspensions of *Brucella abortus* (strain 19). *Nature* **171**: 133.

PROUT, T., HUEBSCHMAN, C., LEVENE, H., and RYAN, F. J. (1953).—The proportions of nuclear types in *Neurospora* heterokaryons as determined by plating conidia. *Genetics* **38**: 518.

RYAN, F. J. (1953).—Competitive suppression of prototrophs. *Nature* **171**: 400.

RYAN, F. J., and SCHNEIDER, L. (1949).—Consequences of mutation during growth of biochemical mutants. *J. Bact.* **58**: 201.

SAZ, A. K., and EAGLE, H. (1953).—The co-killing of penicillin-sensitive and penicillin-resistant bacteria at low concentrations of the antibiotics. *J. Bact.* **66**: 347.

SMITH, H. H., and SRB, A. M. (1951).—Induction of mutations with β-propiolactone. *Science* **114**: 490.

STEPHENS, C. M., and MYLROIE, A. (1953).—Inhibition effects in back-mutation tests with mutants of *Neurospora*. *Nature* **171**: 179.

WESTERGAARD, M., and MITCHELL, H. K. (1947).—Synthetic medium favouring sexual reproduction. *Amer. J. Bot.* **34**: 573.

WITKIN, E. M. (1947).—Mutations in *Escherichia coli* induced by chemical agents. *Cold Spr. Harb. Sym. Quant. Biol.* **12**: 256.

EXPLANATION OF PLATE 1

Fig. 1.—Effect of *ad−3* (W40) conidia on the growth of *ad−3*$^+$ conidia on minimal medium. All three plates contain an equal number of *ad−3*$^+$ conidia and the following numbers of *ad−3* conidia: (*a*) none; (*b*) 2×10^7; (*c*) 4×10^7.

Fig. 2.—The number of *leuc* $^+$ colonies which appear on minimal medium when various numbers of *leuc* (K42) conidia are added to petri plates is compared with the number which arise following the plating of irradiated conidia on a similar medium. The number of *leuc* conidia per plate is: (*a*) 5×10^7, including the 95 per cent. killed by the irradiation; (*b*) 5×10^7; (*c*) 5×10^6; (*d*) 5×10^5; (*e*) 5×10^4; (*f*) 5×10^3.

DETECTION OF MUTATIONS IN MICROBIAL POPULATIONS