DRUG RESISTANCE IN A MUTANT STRAIN OF ASPERGILLUS NIDULANS

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By

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ABSTRACT

The effects of the inhibitors proflavine and phenylmercuric acetate upon germination of conidia of a mutant of Aspergillus nidulans were investigated. Conidia were observed microscopically in the germination process, on media containing various concentrations of the inhibitors. Resistance was induced to gradually increasing concentrations of the inhibitors by continual subculture of the organism in their presence. The effect of this training process upon resistance to the inhibitor, during the various phases of germination and other stages of the asexual life cycle of the organism, were studied at length.

The untrained strain of the mutant proved relatively resistant to proflavine, and a small inoculum could grow on petri plates containing concentrations of the inhibitor up to 400 ppm. This strain could be trained to resist concentrations of proflavine up to 475 ppm on petri plates. The principal effects of proflavine on germination were shown to be inhibition of spore swelling, prevention of the production of a germ tube, and decreased production of mycelium. In growth chambers, strains either trained or untrained to proflavine, germinated almost equally well on concentrations up to 800 ppm, but on 2000 ppm a greater proportion of spores of the resistant strain succeeded in germinating than did those of the untrained strain.

Nearness of spores was found to increase the initial germination rate of both strains. The size of conidia of trained and untrained strains just prior to germination was found to be between 7.1 and 8.2 μ. The majority of those spores which failed to germinate on 2000 ppm proflavine retained a diameter of less than 4.4 μ.

The mutant had been trained to grow on 0.50 ppm phenylmercuric acetate in petri plates. In growth chambers (where the inoculum used was much heavier than that added to petri plates) growth of both trained and untrained strains was obtained on 0.50 ppm. The closeness of spores increased the germination rate. The inhibitor at concentrations higher than 0.50 ppm completely inhibited the swelling of the spores and other phases of germination. Heavy deposits were seen in the hyphae of both trained and untrained strains growing in the presence of phenylmercuric acetate. These deposits were observed to appear much earlier in the hyphae of the resistant strain than in those of the untrained strain. Hyphal growth was retarded in both strains, and sporulation did not occur in the sensitive strain in the presence of the drug. Conidiophores produced by the trained strain growing in the presence of phenylmercuric acetate were considerably shorter than normal, though the conidial heads and conidia appeared normal. The trained strain was found able to overcome the effects of the inhibitor on all phases of the asexual life cycle, after continual subculture in its presence, at concentrations up to 1.0 ppm.

These findings are discussed in the light of the available literature. Two major theories which might explain the attainment of resistance by training, namely enzyme adaptation and gene mutation, are considered. The possibility that phenylmercuric acetate may be absorbed in a non-toxic form by the hyphae of resistant strains is discussed.

INTRODUCTION

The phenomena whereby microorganisms become resistant to the effect of erstwhile inhibitors have been extensively studied, particularly in recent years, when knowledge of microbial genetics has increased. Two schools of thought have offered somewhat opposing explanations for the development of resistance: those who have suggested as the cause, the adaptation of existent enzymes (Dean and Hinshelwood, 1) and those who have frowned upon adaptation, suggesting instead that gene mutations are responsible for resistance (Cavalli and Maccacaro, 2 ; Newcombe 3). Others in the field have suggested that the inhibitor itself acts as a mutagen in the process. Latterly, with the work of Bartlett and Hinshelwood (4), the two opposing views have approached a possible compromise, since it has been shown that both processes (mutation and adaptation) may operate equally well, yet independently of each other.

The list of organisms investigated particularly among bacteria and the various drugs to which they had developed a resistance, is a long one, and apparently every aspect of resistance has been covered. The biochemical implications have been considered. Mutation, adaptation, reversion, inducing agents, specificity, cross-resistance, gene loci, and a host of other topics have been discussed and studied.

Methods have been devised (e.g. replica plating) for discovery of mutants in a growing culture.

Whereas bacteria and yeasts have been utilized in such work, very little has been done in the study of drug-resistance in fungi. Pencillium roqueforti Thom and its response to various drugs: phenylmercuric acetate, proflavine, brilliant green, and sodium azide, have been investigated by Bartlett (5). Roper and Kafer (6) studied the loci of genes responsible for mutations leading to resistance to acriflavine in mutants of Aspergillus nidulans. Russell (7) has investigated a strain of P. roqueforti Thom which had a high level of resistance to phenylmercuric acetate.

It is the contention of the author that the fungi, especially the Penicillia and the Aspergilli present many useful advantages to the investigator of drug resistance. Development of the organism commences (in the vegetative cycle) from the germination of a uninucleate spore, which in turn produces septate hyphae with multinucleate cells. Specialized structures are formed which produce second generation spores. In the asexual or vegetative cycle, then, inheritance of genetic characteristics should be carried from generation to generation without alteration, unless, of course, gene mutation occurs. For this reason, and for others which follow, it was determined to make a study of the effects of

certain drugs on a fungus and the organism Aspergillus nidulans was chosen as the test organism.

Because the spore is relatively large in this organism (3 - 4 μ) and because germination is relatively slow (complete in 4 - 6 hours under normal conditions) the process of germination of the spore can be carefully followed, using microscopic and photomicrographic techniques. The organism selected was a mutant of A. nidulans, generously provided by Professor G. Pontecorvo, and has the added advantage of completing the asexual life cycle in a very short period (36 - 48 hours) at 37°.

The organism used has a further advantage, in that it lends itself to genetic analysis, by the methods of Pontecorvo (8). While no such analysis is to be reported here, it is to be hoped that this phase of the study will be later undertaken.

Since the spores of A. nidulans send out germ tubes after approximately four hours incubation, and growth proceeds fairly rapidly (but not too rapidly) thereafter, the whole cycle of swelling, emergence of germ tube, growth of mycelia, production of conidiophores and conidia can be followed, within a reasonable length of time. Since it can be assumed that only haploid conidia were investigated, it was known that the hyphae, and therefore the conidia produced on sporulation, all contained nuclei with the same genetic structure as the parent conidium.

In these growth chambers, growth of the organism on drug-free medium and drug-containing medium could be followed, the differences noted, and a step made in the direction of finding the effect of any particular drug on the fungal growth cycle. Any number of drugs and their effect on spore size, length of germ tube, production of conidia, etc. can be investigated using growth chambers. Proflavine and phenylmercuric acetate were chosen in the present experiments, because at least some study had already been made with the effects of these drugs on fungi (Bartlett, 5 ; Russell, 6).

MATERIALS AND METHODS

CULTURE OF STRAINS:

The mutant strain paba 1, y, pyro 4 (provided by Dr. G. Pontecorvo) of the wild type Aspergillus (Sterigmatocystis) nidulans (Eidam) Winter, 1884, was used. This mold is an ascomycete of the Order Plectascineae, Family Aspergillaceae. Tests showed that the mutant required para-amino benzoic acid and pyridoxin. The conidia were of a bright yellow colour. When we refer to the sensitive strain, we mean the mutant which had never been grown on proflavine or phenylmercuric acetate; when referring to the resistant strain, we mean the originally sensitive strain, which had either (1) been trained to grow on successively higher concentrations of proflavine, until it produced sporulating colonies on 475 ppm., and which had been maintained on medium containing the drug (either 375 or 400 ppm. proflavine) since at least the latter part of December, 1960; or (2) been trained to grow on 0.50 ppm. phenylmercuric acetate by sub-culture to increasing concentrations of the drug. When inoculated to petri plates containing complete medium plus drug in various concentrations (0.25 - 0.60 ppm.) it was found that only on those plates containing heavy inocula (10^6 conidia) was there any growth of this resistant strain on concentrations higher than 0.30 ppm. (0.35, 0.40 and 0.60 ppm. specifically).

In all cases where spores were required for inoculation to growth chambers the strain (whether sensitive or resistant) was grown on drug-free complete medium just prior to use in any one experiment. Incubation at approximately 37° lasted 37 hours. If a resistant strain was to be used, spores were taken from colonies grown on the drug medium and inoculated on drug-free complete medium plates, to ensure that sporulation would be complete in 37 hours.

MEDIUM:

The medium used is one devised by Dr. G. Pontecorvo (8) and referred to by Roper and Kafer (6). It consists of the following:

Tap water	1000 ml.
Sodium nitrate	6.0 g.
Potassium chloride.....	0.52 g.
Magnesium sulphate ($MgSO_4.7H_2O$)....	0.52 g.
Potassium phosphate (KH_2PO_4)	1.52 g.
Iron and zinc	traces
Dextrose	10.0 g.
Difco Bacto Peptone	2.0 g.
Yeast extract "Yeastrel"	1.0 g.
Casein hydrolyzate	5.0 ml.
Acid and alkali hydrolyzates of yeast nucleic acid	3.0 ml.
Acid and alkali hydrolyzates of thymus nucleic acid ..... (Omitted from medium used herein because thymus nucleic acid was unobtainable.)	2.0 ml.

Peptic and tryptic casein digest ...	5.0 ml.
Hydrolyzed plasma	3.0 ml.
Hydrolyzed corpuscles	3.0 ml.
B vitamin solution	1.0 ml.

All the above, except the B vitamin solution, were mixed together. The pH was adjusted to 6.0 ± 0.2; 15.0 g. of agar were then added. The medium was then autoclaved at 10 lbs. pressure for 10 mins. After sterilization and while the medium was still hot, the B vitamin solution, which had been sterilized previously by the Koch process, was added. The medium was allowed to cool and then placed in a refrigerator.

The various solutions contained in the medium were prepared as follows:

Casein hydrolyzates: 1 liter (or 100 ml.) solution made from 200 g. (or 20 g.) Difco Bacto Casamino acids. Solution will keep in the dark over chloroform for up to three (3) months. (Soluble casein may be used.)

Nucleic Acid Hydrolyzates: 2 g. nucleic acid are placed in 15 ml. 1 N NaOH; 2 g. nucleic acid are placed in 15 ml. 1 N HCL. The

two mixtures are heated at 100°C. for 20 minutes, then mixed, brought to pH 6, and filtered hot. The volume is adjusted to 40 ml. The solution is kept in the dark over chloroform: to be shaken before taking samples.

Casein Digests: 30 g. soluble casein are mixed with 250 ml. water and divided into equal portions. Portion 1 is brought to pH 8 with NaOH and 2 g. trypsin is added. Portion 2 is brought to pH 8 as above and 2 g. pepsin powder are added. Both are incubated at 40° over chloroform for 40 hours. Each is then centrifuged. The supernatants of portions 1 and 2 are boiled and both adjusted to pH 6. The sediments of 1 and 2 are mixed with water to a paste and adjusted to pH 1 with HCl. To the sediment of 1, 1 g. of pepsin powder is added. To sediment of 2, 2 g. of trypsin are added. The sediments are incubated at 40° for 40 hours, adjusted to pH 6, and boiled. All four solutions

are mixed, brought up to 240 ml. and pH 6, and stored in the dark over chloroform.

Hydrolyzed plasma and corpuscles: Plasma from citrated human blood (Dr. Pontecorvo used oxalated horse blood) (25 ml.) was mixed with 25 ml. of 2 N HCl, autoclaved at 120° for 10 minutes, adjusted to pH 7, filtered with suction, brought up to 50 ml. volume, and stored in the dark over chloroform. Corpuscles from the same blood were treated in the same way, but the pH was adjusted to 10, and volume brought up to 75 ml.

Vitamin Solution:

riboflavin	10	mg.
nicotinamide	10	mg.
p-amino-benzoic acid	1	mg.
pyridoxin-HCl	5	mg.
aneurin-HCl (thiamine-HCl)	5	mg.
biotin	0.02	mg.
calcium-pantothenate	20	mg.
choline chloride	20	mg.
inositol	40	mg.
folic acid	1	mg.
distilled water	10	ml.

All the foregoing constituents were mixed together and then Koch sterilized - i.e. the vitamin solution was placed in the autoclave and steamed - for 30 minutes under no pressure.

Two types of experiments were employed:

(1) Petri plates containing either drug-free complete medium or complete medium with various concentrations of the drug, were inoculated, generally with one hundred (100) spores, to determine the colonial growth of the organism in the absence and presence of drug.

(2) This involved the use of the growth chambers (to be described later). These were used to observe microscopically the process of germination of the fungal spores in the presence and absence of the inhibitors.

"Drug-containing" medium

Inhibitors:

When proflavine-containing plates were required either: (1) the required amount of proflavine (3, 6 - diamino acridine monohydrogen sulfate) (National Medical Products; Allied Chem. & Dye Corp.; National Aniline Division) was weighed on

an analytical balance and added to 20 ml. of complete medium or (2) the necessary amount of a solution of proflavine was added to the complete medium, to bring about the required concentration.

When the drug solution was used in the preparation of drug-containing media for petri plates and growth chambers, calculations were made which gave the volume of the solution to be added to complete medium to give a final volume of 20 ml. of medium of the required drug concentration. The calculations were as follows: multiplication of the required concentration of drug by 20 (the desired final volume of drug-containing complete medium), followed by division of the product by the concentration of the drug solution. For example, if the concentration of the drug solution were 3000 µg/ml, then 2.0 ml. of the solution were added to 18.0 ml. of complete medium, to give 20 ml. of medium of a concentration of 300 µg/ml (ppm). Calculations for the above example are:

$$\frac{300 \times 20}{3000} = 2.0 \text{ ml.}$$

The drug solution was made as follows: 1 litre of phosphate buffer, containing 7.765 g. K_2HPO_4 and 2.96 g. KH_2PO_4, was prepared. This was sterilized at 15 lbs./in^2 pressure for 15 minutes. The desired amount of proflavine

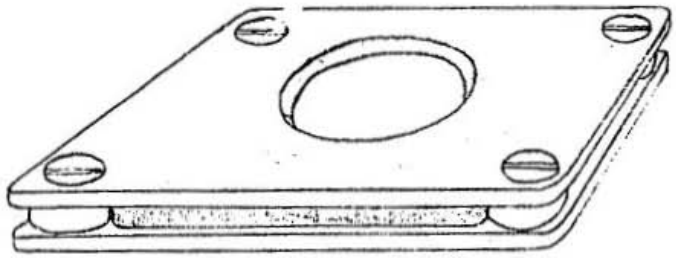
was then dissolved in the still hot, sterile phosphate buffer. The solution was kept in the dark.

When phenylmercuric acetate plates were required, the necessary amount of a solution of this compound (Phenylmercuric acetate (Practical)) (Eastman Organic Chemicals, Distillation Products Industries, Rochester, N.Y., Division of Eastman Kodak Company) was added to the complete medium, to bring about the required concentration (as for proflavine.) The drug solution was made in the same way as the proflavine solution and was kept in the dark.

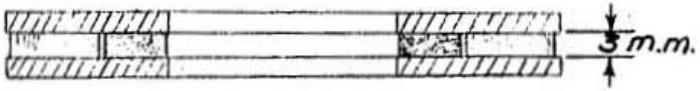
STERILIZATION OF EQUIPMENT: Except where otherwise indicated, sterilization was carried out in an autoclave, kept at a pressure of approximately 15 lbs./in^2, for 15 minutes.

GROWTH CHAMBERS: Two types of growth chambers were used, one suggested by Dr. Burton of the University of Montreal (personal communication), the other designed by Dr. Paul of Glasgow University. (9) The latter offered an opportunity to humidify the agar-containing chamber during growth. Sketches of the two chambers are given in Fig. 1.

Growth Chambers for Cinemicrographic Studies



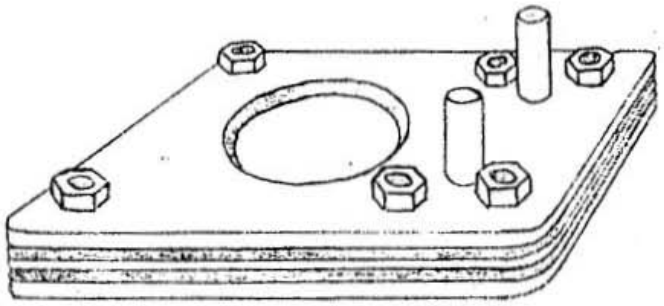
Assembled Chamber



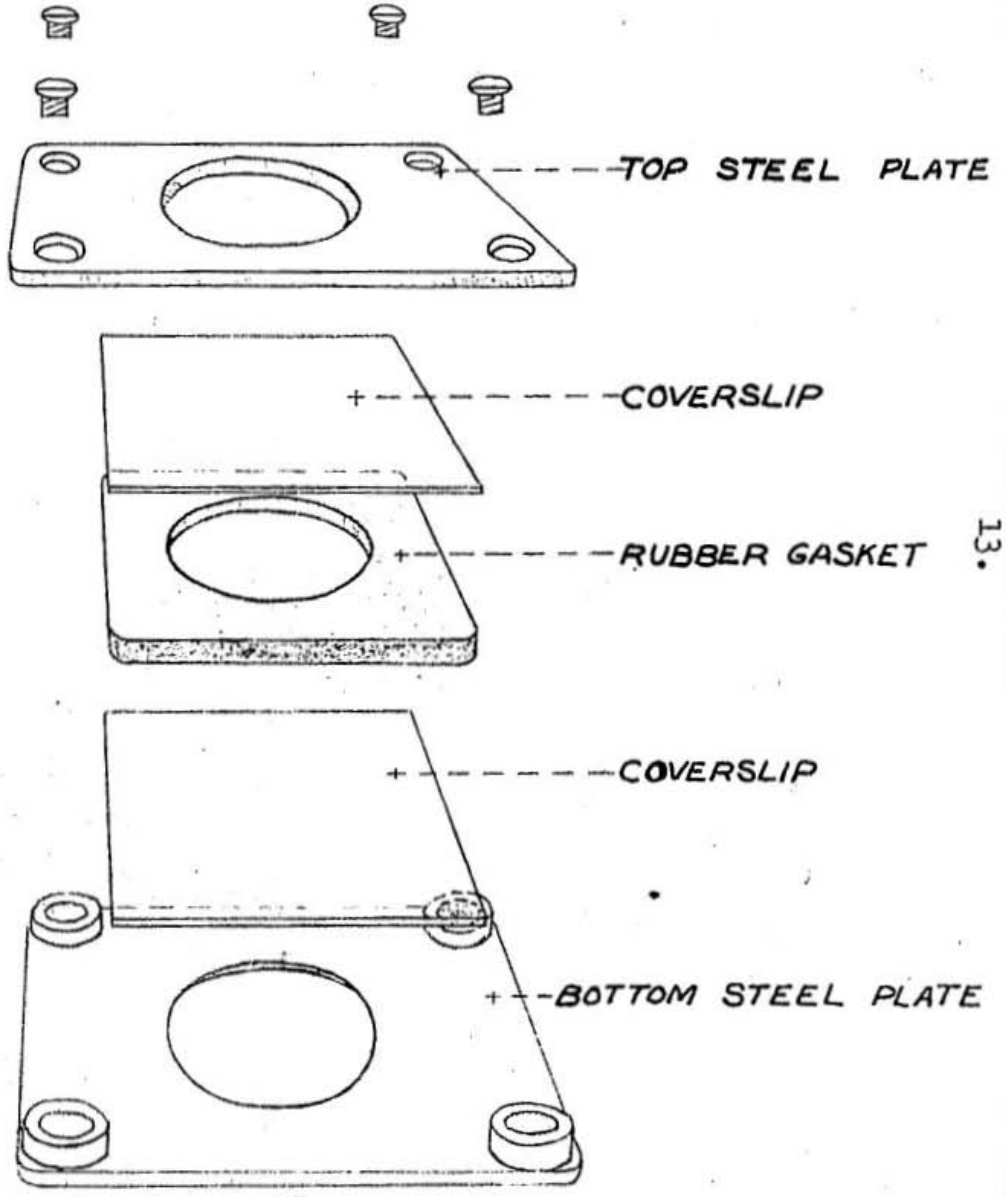
Cross section of Side View
Note: Depth of chamber 3 m.m.

(After design of BURTON)

(After design of PAUL)



Assembled Chamber



"Exploded" Chamber

Fig. 1

The two steel plates of the growth chamber were never sterilized, but were washed with soap and water, rinsed with tap water, and then with distilled water. This was to ensure that no medium was still adhering to the surface of the metal, to interfere with the results of the next experiment.

The cover slips were polished (gently) with lens paper, and then wrapped in lens paper, and placed in a large petri dish. The dish was then placed (covered) in the autoclave and sterilized.

The rubber squares were washed with soap and water, then rinsed with tap water, then distilled water, and placed in another large petri dish, which was then covered. This was sterilized. Again, the washing was carried out to ensure that no medium or drug still adhered to the rubber.

After each experiment was completed, the screws of the growth chamber were loosened (but not removed) and the chamber placed

in the autoclave for the usual 15 minutes. When removed from the sterilizer, the cover slips were discarded and the processes outlined above were carried out, in preparation for the next experiment.

It was found better to allow the sterilized cover slips and rubber squares to dry in the incubator (at approximately 37°) before use. If the rubber squares were permitted to dry on their own, and were then used in a growth chamber, it took a longer time for the spores to come to rest on the medium. It was considered that the rubber must keep some moisture within it and this added to the liquid in which the spores were inoculated, was too much to be dried out in the usual hour's wait before the first count. When spores were moving, not only could they not be measured, but no accurate count could be made since they were continually migrating to other parts of the medium.

Equipment used in assembling of growth chambers:

The floor of the bottom half of a large petri dish was covered with paraffin to a depth of about 5 mm., and two pieces of glass rod (hollow) were laid in the paraffin, at a distance from each other which would allow the two parallel edges of the cover slips to rest one on each rod.

Two cover slips could be accommodated with such an arrangement. These petri plates were used after the cover slips had been dipped in the medium, and needed some place in which to rest while the medium hardened. The cover slips could be taken from these plates when needed for the growth chamber. The medium-covered slips were made up immediately before use - otherwise drying of medium occurred, and results obtained from the experiment in which they were used could not be depended upon.

Several such large petri dishes were prepared from time to time, as the need arose for separate ones in which to place cover slips coated with drug-free or drug-containing medium. In this way it was hoped to avoid the possibility of drug-free medium taking up some drug left by a cover glass covered by drug-containing medium.

These large petri dishes were not always, but usually, sterilized between experiments.

Assembly and inoculation of Growth Chambers:

The complete medium (with or without drug, as required) was melted and poured into a sterile petri plate. Sterilized cover slips were removed from their lens paper covering by means of suction through an unsterile rubber hose attached to a vacuum pump. The

sterile side of the cover glass was then placed in contact with the liquid medium and the excess medium permitted to drop back into the sterile container from the cover slip. The cover slip was then placed in the large sterile petri dish described previously.

Spore suspensions were made by taking either one or several loopfuls of spores from the 37-hour culture mentioned above, and placing these in 3 ml. of 0.85% sterile saline. It had been found that ST37 (Merck, Sharpe & Dohme) a "wetting agent" used to reduce surface tension, prevented germination of the spores, and thus could not be used to help keep the spores apart in the sterile saline suspension. The spore suspension was then pipetted approximately 150-200 times, with a 1 ml. pipette, to separate the spores.

The cover slip containing the medium was then placed, medium side up, on the lower metal plate of the growth chamber, the rubber square put in place, a drop of the spore suspension placed in the centre of the cover slip, and another sterile cover slip placed on the rubber square. The upper metal plate was then placed on top, and the screws put in place. The growth chamber was then incubated in the position in which it has been assembled. After approximately one hour, the growth chamber was turned

upside down, and placed on the stage of the microscope, for observations to be made under a magnification of 400x. A Leitz Ortholux microscope equipped with a phase contrast system was used; the microscope was housed in an incubator chamber ensuring that the spores were kept at the proper temperature (approx. 37°) during counts and measurements.

Counts:

If the average size of the spores was to be taken, two hundred single spores were measured, using a micrometer eyepiece. The micrometer was divided into ten segments, each of which was again divided into ten smaller segments. Each of the segments measured 0.01 mm.

If the per cent germination was to be found, two hundred single spores were counted. If of these 200, say 50 were germinating, the per cent germination would be 25%. If germination per cent and average size were measured at the same time, a total of 200 spores altogether were taken into account. Once germination started, the germinating spore was not measured, but counted among those already germinating. The average size of the non-germinated spores could be found by division of the total of the measurements by the total number of spores actually measured.

In most cases, note was taken of the relative number of groups and single spores present in each growth chamber.

When counts and measurements were made, it was found best to start at some point near the edge of the cover slip, and continue in a straight line across the cover slip until the opposite side was reached. The chamber was then moved, so that a count could be made a small distance below where the last count was made. In this way, 200 spores were counted, and none counted twice.

When time came for the next count, the same procedure was followed, so that the same spores were being followed in their course.

For inoculation of a known number of spores to plates containing medium, a spore suspension was made up in sterile saline, a drop added to the grid of the counting chamber of a haemocytometer, and a count made. After the number of spores per ml. had been calculated from this count, appropriate dilutions were made of the original suspension, to give a final dilution which contained the required number of spores per 0.2 ml. When, therefore, it is stated herein that 100 conidia were inoculated, this number was based on actual count of an original spore suspension.

When growth chambers were used, although no accurate count was made, it can be estimated that approximately 5×10^4 conidia were inoculated to each chamber.

CHAPTER ONE

THE EFFECT OF PROFLAVINE ON THE GERMINATION OF SENSITIVE AND RESISTANT STRAINS OF THE MUTANT.

Normal Growth of Sensitive and Resistant Strains on Drug-Free Complete Medium.

ON PETRI PLATES:

When conidia of the sensitive strain were plated to drug-free complete medium, normal growth occurred on incubation. The first colonies were visible after about 14 hours incubation. At this time each colony was small and consisted of white hyphae radiating from a central point. When growth was allowed to continue, the colonies gradually increased in diameter and started to sporulate. Good sporulation was obtained after approximately 37 hours incubation. At this time the colonies were circular, with a central area of bright yellow conidia, and white hyphae radiated from the outer edge of this yellow area.

When conidia of the resistant strain were plated to drug-free complete medium, the first colonies were apparent after 17-18 hours incubation. Sporulation started after approximately 30 hours incubation and was complete after a further 10-13 hours. The colonies were similar to those produced by the sensitive strain. Upon sporulation the colonies had an average diameter of approximately 12 mm.

(inclusive of hyphae). The spores were bright yellow.

GROWTH CHAMBERS: - Observation of Germination -

From the data in Table 1 and Figures 2 and 3, it can be seen that on drug-free complete medium, both the sensitive and resistant spores start to send out their germ tubes after approximately four hours incubation. In those chambers containing the sensitive spores, when moderate or few groups were present, germination rate was high. Where many groups were present, the rate was low. However, whether many or few groups were present, germ tubes appeared at approximately the same time. Among those chambers containing the resistant spores, there was one in which there were few groups present, and the single spores were far apart. Here per cent germination was lower than in the other three chambers.

The curves rise linearly for both the sensitive and resistant strains. From the slope and height of the graphs it can be seen that the rate of germination was approximately the same for both strains but the final per cent germination of the resistant strain was lower than that of the sensitive strain.

Level of Resistance of the Sensitive Strain to Proflavine:

ON PETRI PLATES: Colony Formation

When 100 conidia (counted by use of a haemocytometer)

TABLE 1

SENSITIVE STRAIN ON DRUG FREE COMPLETE MEDIUM*

GROWTH CHAMBER	# 1	# 2	# 3	# 4
RELATIVE NUMBER OF SINGLE SPORES	Not Noted	Not Noted	Not Noted	Not Noted
RELATIVE NUMBER OF GROUPS	Moderate	Many	Few	Few
DURATION OF INCUBATION (Hrs.)	PERCENT GERMINATION			
4	8.0	6.5	12.5	3.5
6	100.0	64.0	92.5	90.5
9	100.0	67.0	98.0	96.5

RESISTANT STRAIN ON DRUG FREE COMPLETE MEDIUM*

GROWTH CHAMBER	# 1	# 2	# 3	# 4
RELATIVE NUMBER OF SINGLE SPORES	Not Noted	Not Noted	Spores Far Apart	Not Noted
RELATIVE NUMBER OF GROUPS	Moderate	Moderate	Few	Very Few
DURATION OF INCUBATION (Hrs.)	PERCENT GERMINATION			
4	19.5	3.0	3.5 ▲	0.5
6 1/2	90.5	89.0	72.5	83.0
10	95.0†	98.0	84.5 △	91.5 △

*The spores for each growth chamber were produced by a different colony.
†In some places hyphae were so far advanced that they may have hidden some single spores.
▲Difficult to focus, since the spores were sunken into the agar - count may be incorrect.
△Growth of hyphae not extensive.

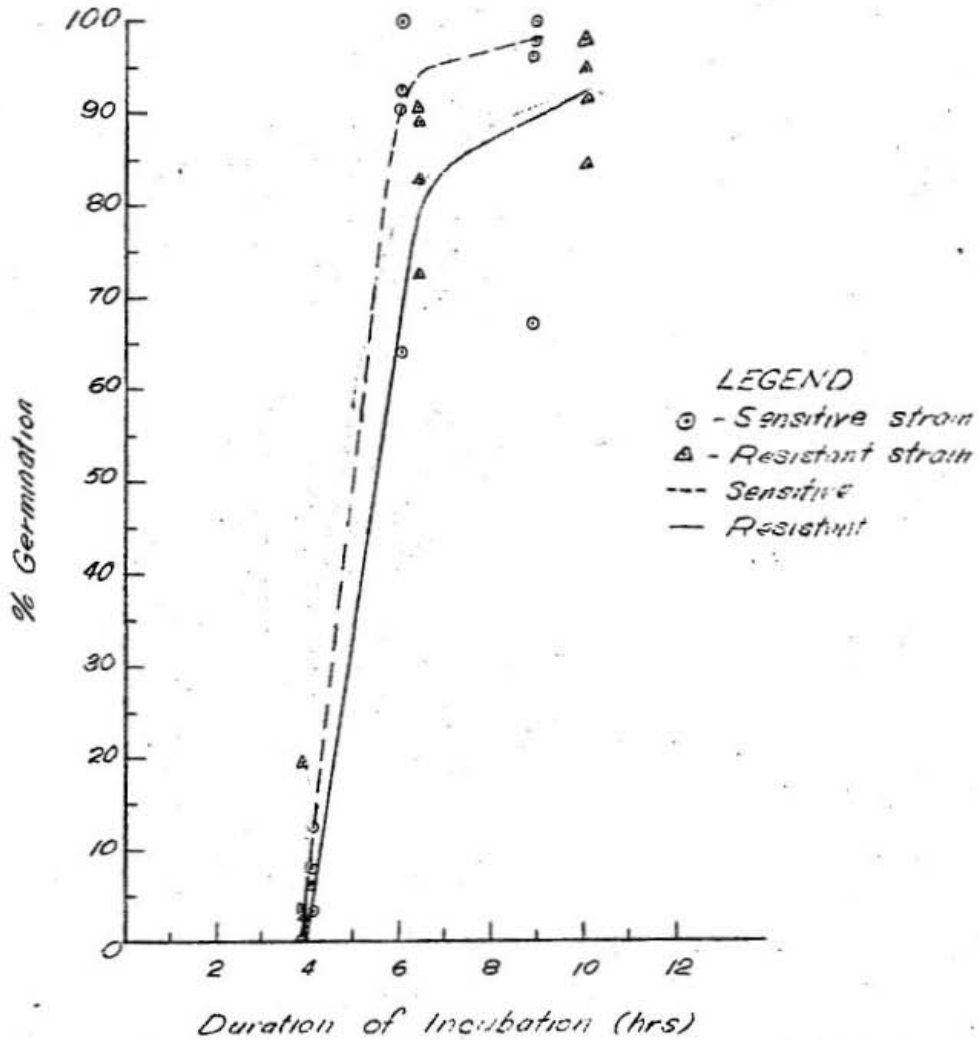


Fig. 2 Graphical Representation of Data in Table 1 (Sensitive and Resistant Strains on drug-free Complete Medium).

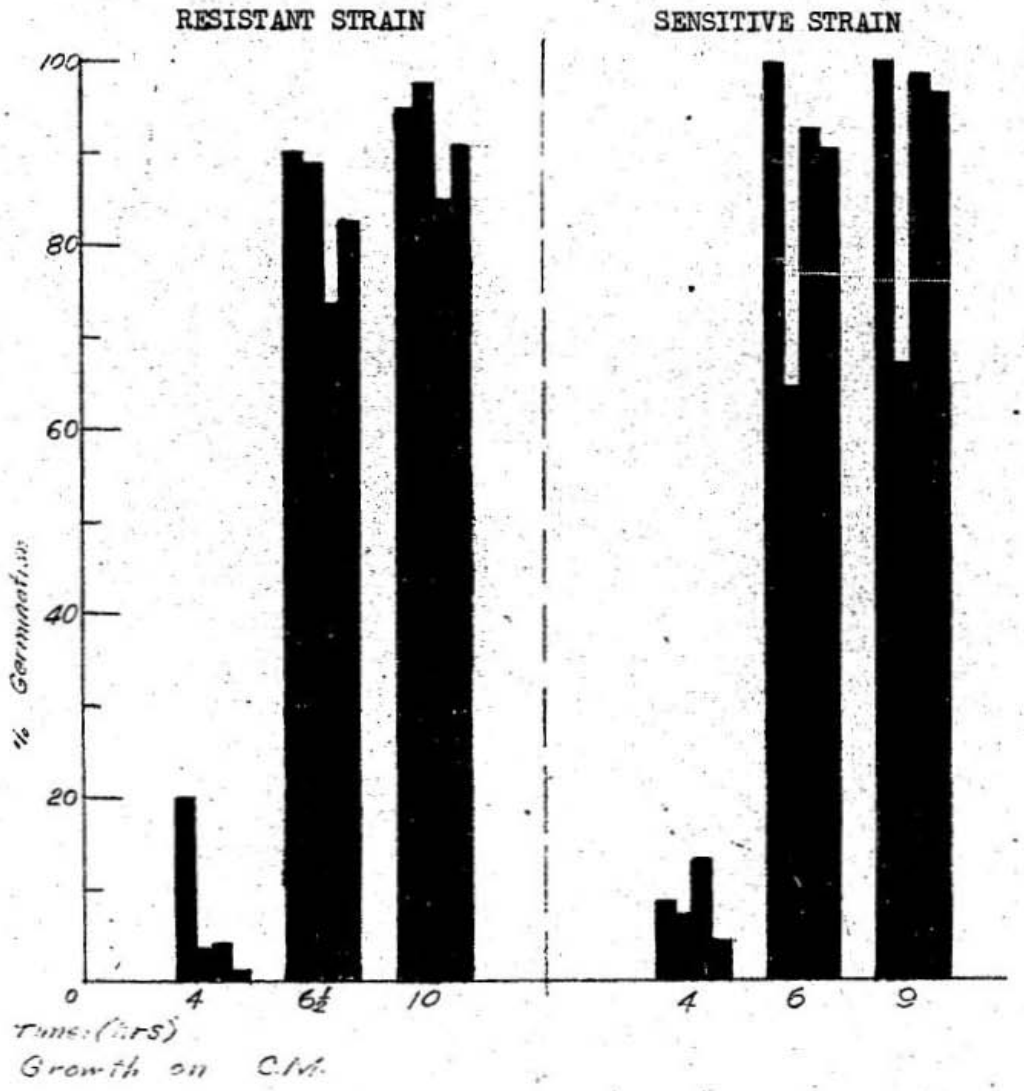


Fig. 3 Histogram showing germination per cent of sensitive and resistant strains on drug-free complete medium after various periods of incubation in growth chambers.

of the sensitive strain were plated directly on to complete medium plates containing proflavine in concentrations up to 350 ppm, growth occurred. At times growth was obtained on 375 and 400 ppm.

On drug plates, appearance of colonies was delayed. In some cases, long periods existed between readings, and therefore the exact time of appearance of colonies is not known.

There was fairly close agreement among the results of the three tests of sensitivity to proflavine. On concentrations of up to 225 ppm, colonies appeared after approximately 20 hours incubation. There was some difference in the results on 250 and 300 ppm, colonies appearing after 20 hours in one test series and 37 hours in another. Since the plates in the third test were not read until after 45 hours incubation, no readings from that series are considered except those for plates containing drug in concentrations greater than 300 ppm. In two only of the three test series did the range of concentration include 325 to 400 ppm proflavine. These two tests were run in quintuplicate. The time of appearance of colonies on 325 and 350 ppm were approximately the same for both tests: after 45 and 48 hours on 325 ppm, and after 68 and 72 hours on 350 ppm. However, on 375 and 400 ppm, growth occurred in only one of these two tests: that on 375 ppm after 91 hours and that on 400 ppm after 112 hours; no growth occurred on these concentrations in the other test, even after 96 hours incubation. The results of the three tests are shown graphically in Fig. 4.

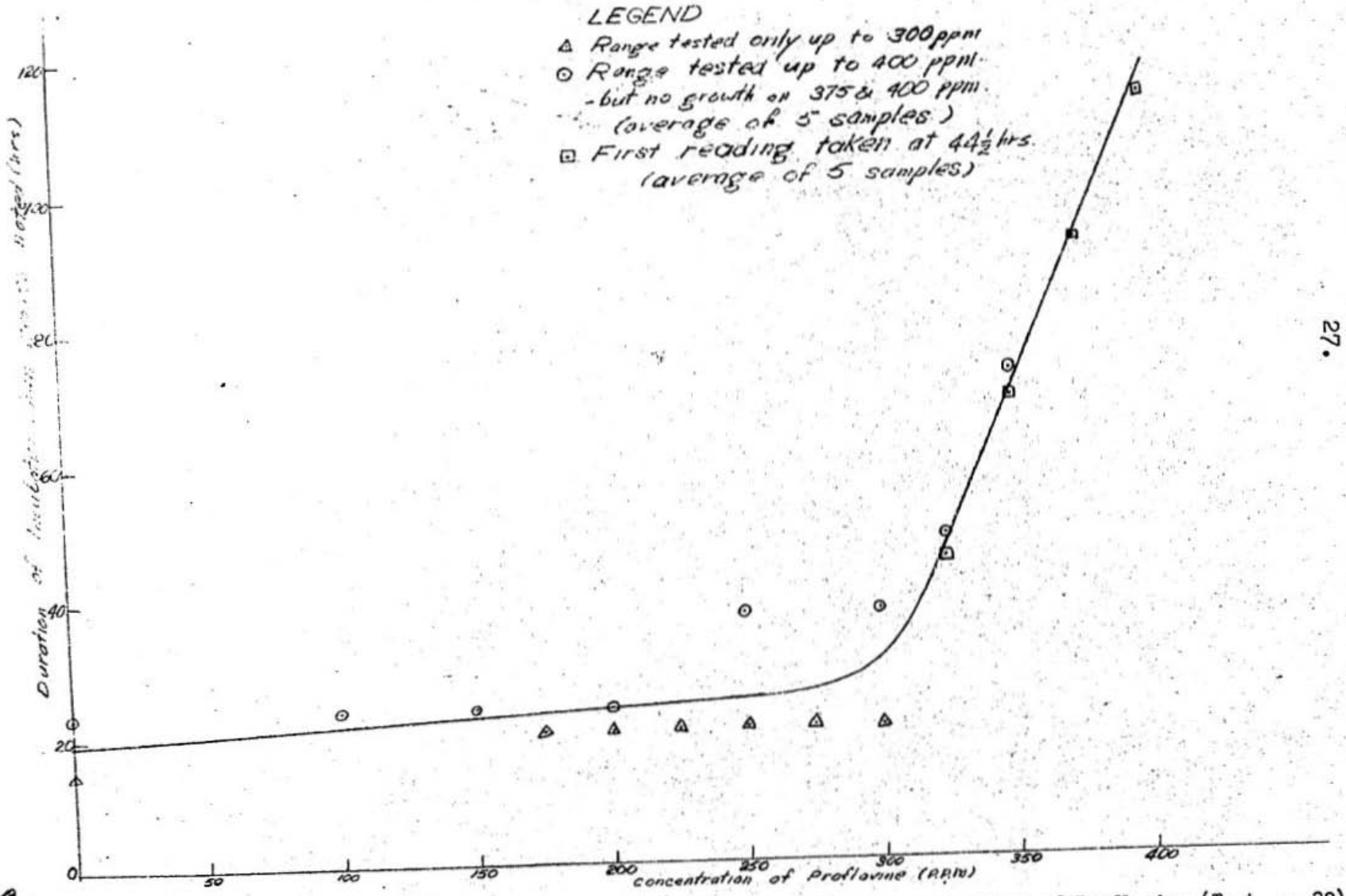


Fig. 4 Time of appearance of colonies of Sensitive Strain on various concentrations of Proflavine (Text, p. 22)

On concentrations of the drug up to 150 ppm, the number of colonies was similar to that on drug-free complete medium. On 125 ppm and above, the size of colonies diminished with increased drug concentration. On 175 ppm and higher, the number of colonies diminished with increasing concentration.

Hyphae of colonies on drug-free and drug-containing plates were at first white, but on extended incubation, hyphae of colonies on drug plates gradually became yellow or buff-coloured, probably from absorption of proflavine whereas the hyphae of colonies on drug-free plates remained white. On the highest concentrations (350-400 ppm) the colonies were so confined by the medium that hyphae were not prominent.

Colonies on these high concentrations were confined from the beginning. After prolonged incubation, colonies on lower concentrations (250-300 ppm) became confined, and colonies on these concentrations and higher became raised. On 350-400 ppm plates, at times, colonies were found which were wholly yellow, confined and raised. These were tough and leathery.

Sporulation occurred at about 20 hours after appearance of colonies, whether on drug-free or drug-containing medium.

Conidia on sporulating colonies were at first bright yellow, but on aging turned a mustard colour.

Level of Resistance of Resistant Strain to Proflavine:

ON PETRI PLATES:

Although the resistant strain had been trained by a laboratory colleague (unpublished) to higher and higher concentrations of proflavine, until the strain produced colonies on 575 ppm proflavine, further testing of this strain on such high concentrations showed that it was not resistant to this concentration: In fact growth could not be obtained on concentrations of the drug above 475 ppm. This loss of resistance may be explained by the fact that the strain was kept in the refrigerator for some months after training and before being used in the within experiments. The results of three of the tests carried out to find on what concentrations sporulating colonies could be grown are given graphically in Fig. 5 . Level of resistance is therefore defined herein as that concentration of drug-containing complete medium on which sporulating colonies will be produced by the strain.

Since the results of the three tests were too close to justify the drawing of three graphs, only one is given. However, the points taken from the results are given separately and represented by three different symbols, for comparison's sake. The actual line is formed by joining points which represent the average number of colonies appearing on each concentration. If A, B and C represent

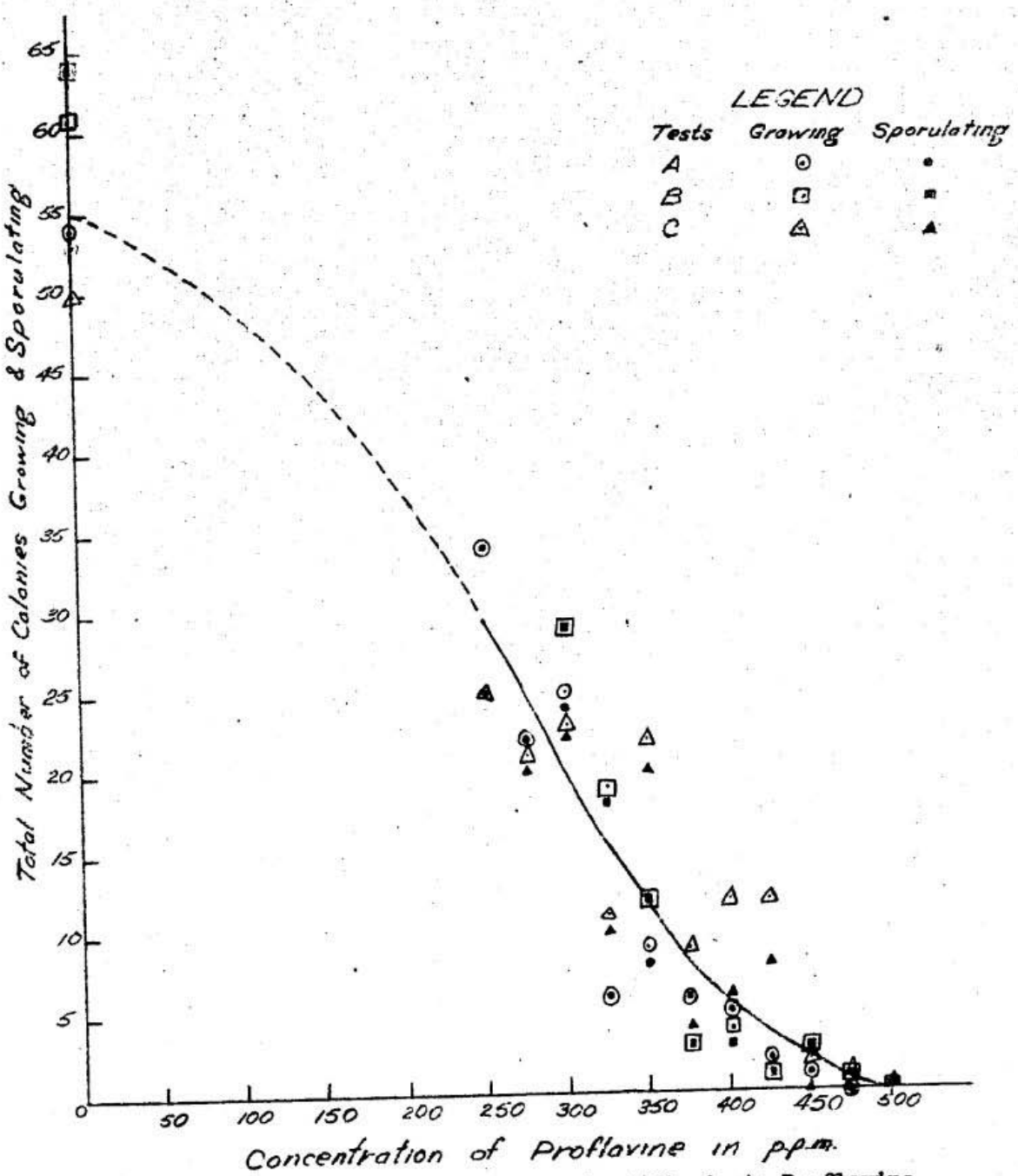


Fig. 5 Level of Resistance of Resistant Strain to Proflavine.
(See Text, p. 29)

the three tests, the history of the conidia prior to the tests and the range of drug concentrations used in the tests are given below:

A. 100 conidia from the control plate (drug-free complete medium) of a subculture from the 425 ppm plate of a prior subculture from the original resistant strain were inoculated to drug-free complete medium, and a series of complete medium plates containing 275-500 ppm proflavine.

B. 100 conidia from the 300 ppm plate of A were inoculated to drug-free complete medium, and a series of complete medium plates containing 300-500 proflavine.

C. 100 conidia from the 400 ppm plate of a subculture from the 425 ppm plate of B were inoculated to drug-free complete medium, and a series of complete medium plates containing 250-500 ppm proflavine.

The maximum growth and sporulation time for:

A. was approximately 137 hours.

B. was approximately 83½ hours.

C. was approximately 64½ hours.

The curve shows that only about 57% of the conidia inoculated germinated on drug-free complete medium, and only about 50% of that number succeeded in germinating on 300 ppm proflavine.

The three strains grew equally well on concentrations of proflavine up to 300 ppm., and were equally resistant to concentrations of proflavine of 450-475 ppm. All three failed to germinate on 500 ppm.

Of those conidia which germinated, all produced colonies with normal sporulation on drug-free complete medium. Nearly all of the conidia which germinated produced normal sporulation on concentrations of proflavine up to 350 ppm.

On all concentrations of the drug, the colonies were confined, and when growth was allowed to continue, the colonies became more confined and raised. Some colonies became conical. On the higher concentrations (350-450) the colonies were smaller and more confined than on lower concentrations; however, after about 137 hours incubation, the hyphae of those colonies on 375-450 ppm began to grow beyond the region where the colony edge had sunken beneath the level of the surrounding medium.

Initially, the conidia were bright yellow, On prolonged incubation they became a mustard colour. Similar observations were made on drug-free complete medium. The hyphae were at first white and remained so on drug-free medium, but became yellow or buff-coloured on lengthy growing on drug-containing medium. They probably took this colour from absorbed proflavine.

Level of Resistance of Sensitive and Resistant Strains to Proflavine.

IN GROWTH CHAMBERS: Observations of Germination

The phases of germination referred to earlier (swelling of the conidium, emergence of the germ tube, and extension of the germ tube) were followed by means of growth chambers, and phase contrast microscopy. Where germination is referred to herein, it implies the emergence of the germ tube. Other observations were made of the phasesof swelling and extension of the germ tube, but these have not been taken into account in most of the descriptions of results.

At the outset, no consistent set of results could be obtained from similar growth chambers, perhaps due to variations in the medium, brought about by the method initially used of preparing growth chambers. Although the results are not tabled here, an early experiment showed that in two growth chambers containing sensitive spores on 100 ppm proflavine, germination did not begin until 9½ hours after incubation began; after thirteen hours incubation, there was 61% germination in one chamber, and 21% in the other.

The results of two other chambers, containing resistant spores on 800 ppm proflavine, showed an even wider disparity. In one, germination had not begun at 10 hours, and after 24½ hours incubation, there was only 3% germination. In the other, germination began at approximately 6½ hours and had

progressed to 30.5% after approximately 10 hrs incubation.

Because of these inconsistencies, the following procedure was adopted and was found to give more consistent results: four growth chambers were prepared (as were described earlier); to permit time for a count to be made on each at approximately the same time, the growth chambers were prepared at half-hour intervals. Each contained exactly the same preparation of medium and was inoculated from the same spore suspension, in most cases obtained from a single colony. This would avoid the consequences of any difference in resistance of the conidia, and assure consistency in experimental method.

From preliminary experiments, it seemed possible that variations existed in the level of resistance of different colonies from the same culture. To test this possibility, four complete medium plates were inoculated with spores from four different colonies on the same culture plate. Four growth chambers were inoculated with conidia from a different complete medium plate. Thus, each chamber received conidia derived from a different conidium. The results of these growth chambers are given in Tables 1 and 2 . The results within each set are similar.

Emergence of germ tubes had been found to begin after approximately four hours incubation and since only data on the rate of germination and per cent germination were looked for, this was the period at which the first count was made.

For the sake of easy comparison, times approximate to the nearest hour or half hour are given.

Nearness of spores was found to affect germination. For this reason, only single spores and not those within groups were counted. The relative number of groups was noted, since early germination in these affected nearby single spores. As the experiments progressed, the relative number of single spores was also taken into account, since this too affected germination rate. The effect of nearness was to increase the rate of germination of the conidia on drug-containing complete medium. Two hundred conidia were checked at each interval.

The results are given below in Tables 1 - 5 . The results for sensitive and resistant strains on the same medium are given within the same table . Figures 2,6-9 represent graphically the results from Tables 1 - 5 respectively. The graph is drawn by use of the points obtained by taking the arithmetic mean of the typical groups. This is termed the modified mean or Modal. Although these calculated points do not appear on the graphs, the actual results of the experiments are shown, to give a true picture.

The results for the sensitive and resistant strains on drug-free complete medium have already been referred to (see Fig. 2 , p. 24).

On complete medium containing 100 ppm proflavine (Table 2 Fig. 6), the germ tube of the resistant and sensitive strains emerged at approximately 4½ hours after commencement of incubation. The number of single spores present was not noted. Among those chambers containing the resistant conidia, the germination rate was lower where few, rather than many or a moderate number of groups, were present. Where there was a moderate number of groups present, rather than very few, the sensitive strain exhibited a much higher initial rate. As the incubation period lengthened, however, approximately the same number of spores were germinating in all four growth chambers containing the sensitive strain.

It can be seen that there was very little difference in the effect of the drug on the two strains, whether trained to the proflavine or sensitive. The rate of germination was the same, but the final per cent germination was a little lower for the sensitive than for the resistant strain.

On complete medium containing 600 ppm. (Table 3 ; Figure 7), the germ tubes of the resistant strain became apparent after approximately 4 hours incubation, and the sensitive strain after approximately 4½ hours. The graphs show a constant increase in the number of sensitive spores with germ tubes. The resistant curve continues to rise

TABLE 2

SENSITIVE STRAIN ON COMPLETE MEDIUM CONTAINING 100 ppm PROFLAVINE*

GROWTH CHAMBER	# 1	# 2	# 3	# 4
RELATIVE NUMBER OF SINGLE SPORES	Not Noted	Not Noted	Not Noted	Not Noted
RELATIVE NUMBER OF GROUPS	Very Few	Very Few	Very Few	Moderate
DURATION OF INCUBATION (Hrs.)	PERCENT GERMINATION			
4 1/2	9.0	10.0	5.0	29.5
6 1/2	86.0	80.5	80.0	90.0
9	91.5	89.5	87.5	96.5

RESISTANT STRAIN ON COMPLETE MEDIUM CONTAINING 100 ppm PROFLAVINE*

GROWTH CHAMBER	# 1	# 2	# 3	# 4
RELATIVE NUMBER OF SINGLE SPORES	Not Noted	Not Noted	Not Noted	Not Noted
RELATIVE NUMBER OF GROUPS	Moderate	Moderate	Few	Many
DURATION OF INCUBATION (Hrs.)	PERCENT GERMINATION			
4 1/2	8.0	10.0	2.5	10.5
6 1/2	86.0	88.0	72.0	95.0
10	92.0†	95.0†	80.0	93.0†

*The spores for each growth chamber were produced by a different colony.
†In some places hyphae were so far advanced that they may have hidden some single spores.

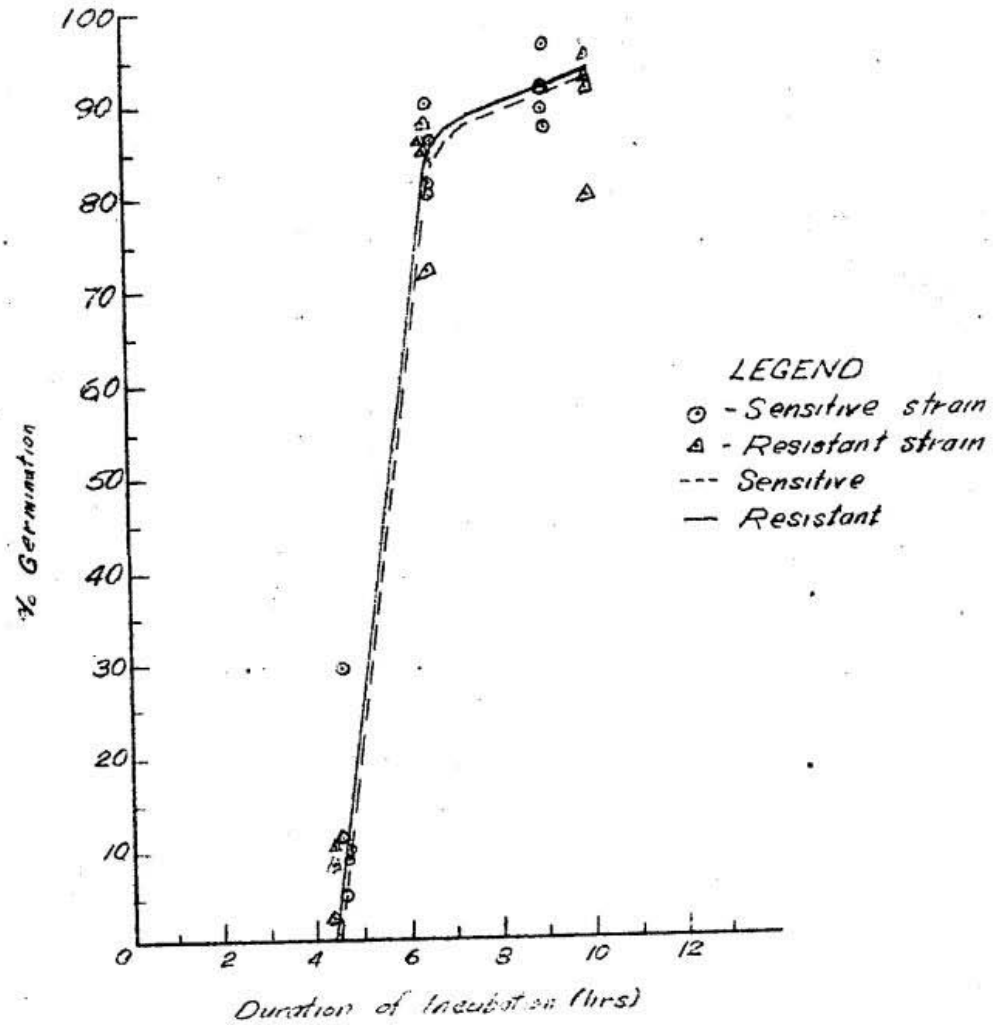


Fig. 6 Graphical Representation of Data in Table 2 (Sensitive and Resistant Strains on Complete Medium containing 100 ppm Proflavine).

TABLE 3

SENSITIVE STRAIN ON COMPLETE MEDIUM CONTAINING 600 ppm PROFLAVINE

GROWTH CHAMBER	# 1	# 2	# 3	# 4
RELATIVE NUMBER OF SINGLE SPORES	Not Noted	Not Noted	Not Noted	Not Noted
RELATIVE NUMBER OF GROUPS	Moderate	Moderate	Moderate	Moderate
DURATION OF INCUBATION (Hrs.)	PERCENT GERMINATION			
4 1/2	0	0	Not Read	Not Read
5	1.5	2.5	4.5	11.5
8	36.0	51.0	49.0	55.0
10	66.0	67.5	69.0	73.5
12	Not Read	Not Read	Not Read	Not Read

RESISTANT STRAIN ON COMPLETE MEDIUM CONTAINING 600 ppm PROFLAVINE

GROWTH CHAMBER	# 1	# 2	# 3	# 4
RELATIVE NUMBER OF SINGLE SPORES	Very Many	Very Many	Very Many	Very Many
RELATIVE NUMBER OF GROUPS	Moderate	Moderate	Moderate	Moderate
DURATION OF INCUBATION (Hrs.)	PERCENT GERMINATION			
4	0	0	0	0
6	6.5	22.0	26.0	24.0
8 1/2	43.5	4[illegible].5	63.0	71.5
10	54.0 •	72.0	75.0 †	79.5 †
12	52.0 •†	79.5 †	83.0 †	79.0 †

• Difficult to see single ungerminating spores, since present at two levels of medium.
† In some places, hyphae so far advanced that they may have hidden some single spores.

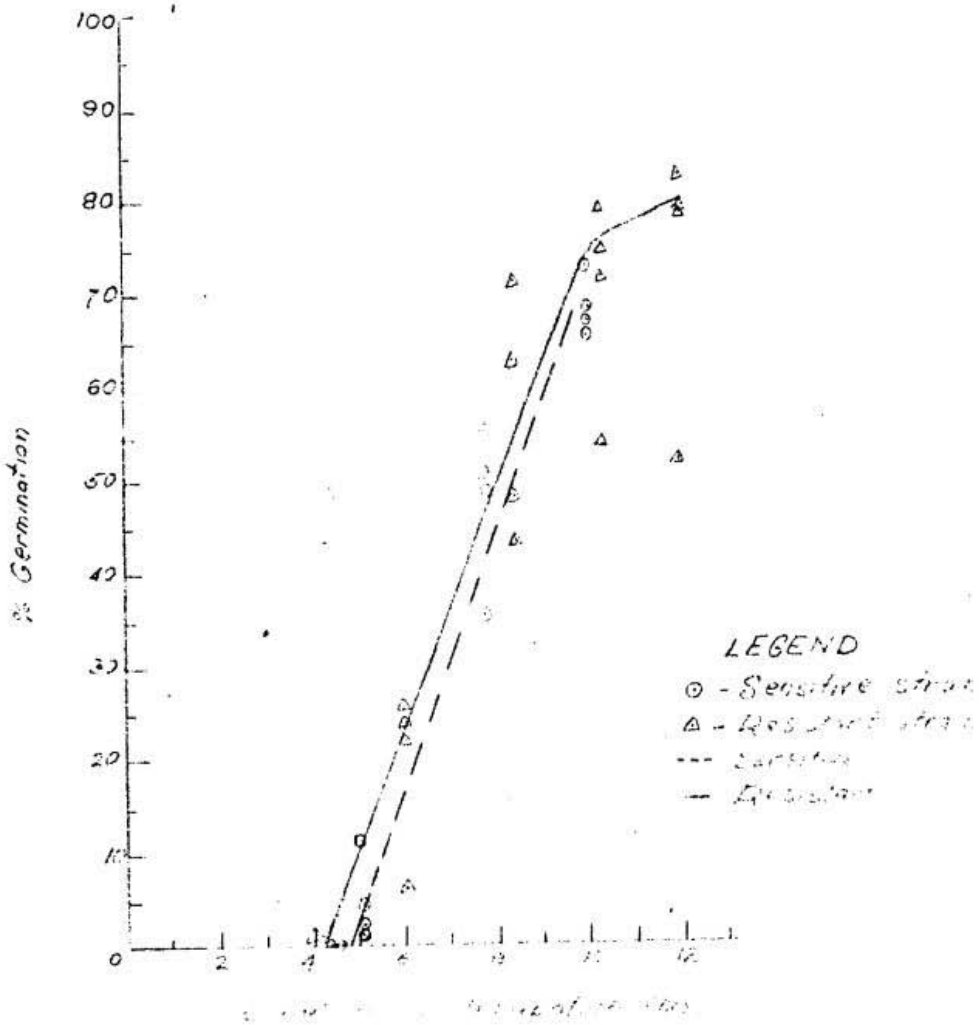


Fig. 7 Graphical Representation of Data in Table 3 (Sensitive and Resistant Strains on Complete Medium containing 600 ppm Proflavine).
N.B. One point may represent two or more identical readings - see Table 3.

linearly until approximately 10 hours, when it begins to flatten. Once started, the sensitive strain germinated a little faster than the resistant.

On complete medium containing 800 ppm. (Table 4; Figure 8), both strains evidenced germ tubes at approximately the same time (about 4½ hours). Although two of the growth chambers containing the sensitive spores contained few groups, there was seemingly no adverse effect brought about by this circumstance.

The curves for both strains rise linearly. From the angle of the graphs, the sensitive appear to be germinating at a faster rate than the resistant, but the sensitive strain did not achieve as great a total germination.

On complete medium containing 2000 ppm. (Table 5; Figure 9), when many spores were present, the resistant and sensitive strains started sending out germ tubes at approximately the same time (4 hours); but when only few single spores were present, conidia of the sensitive strain did not produce germ tubes until after 6 hours incubation. Although the number of sensitive spores present affected the initial rate of germination, the final per cent germinating was approximately the same.

The resistant curve rises linearly to approximately 10

TABLE 4

SENSITIVE STRAIN ON COMPLETE MEDIUM CONTAINING 800 ppm PROFLAVINE

GROWTH CHAMBER	# 1	# 2	# 3	# 4
RELATIVE NUMBER OF SINGLE SPORES	Not Noted	Not Noted	Not Noted	Not Noted
RELATIVE NUMBER OF GROUPS	Very Few	Moderate	Very Few	Moderate
DURATION OF INCUBATION (Hrs.)	PERCENT GERMINATION			
5	5.0	1.5	1.0	7.0
/	/	/	/	/
8	32.0	21.5	20.0	39.5
10	45.0	42.5	33.5	49.0
12	Not Read	Not Read	Not Read	Not Read

RESISTANT STRAIN ON COMPLETE MEDIUM CONTAINING 800 ppm PROFLAVINE

GROWTH CHAMBER	# 1	# 2	# 3	# 4
RELATIVE NUMBER OF SINGLE SPORES	Very Many	Very Many	Very Many	Very Many
RELATIVE NUMBER OF GROUPS	Few	Few	Few	Few
DURATION OF INCUBATION (Hrs.)	PERCENT GERMINATION			
4	0	0	0	0
6	4.5	5.0	16.0	21.0
8	25.5	30.0	40.5	29.5
10	36.0	47.5	50.0	49.5
12	54.5	55.0	56.0	52.0

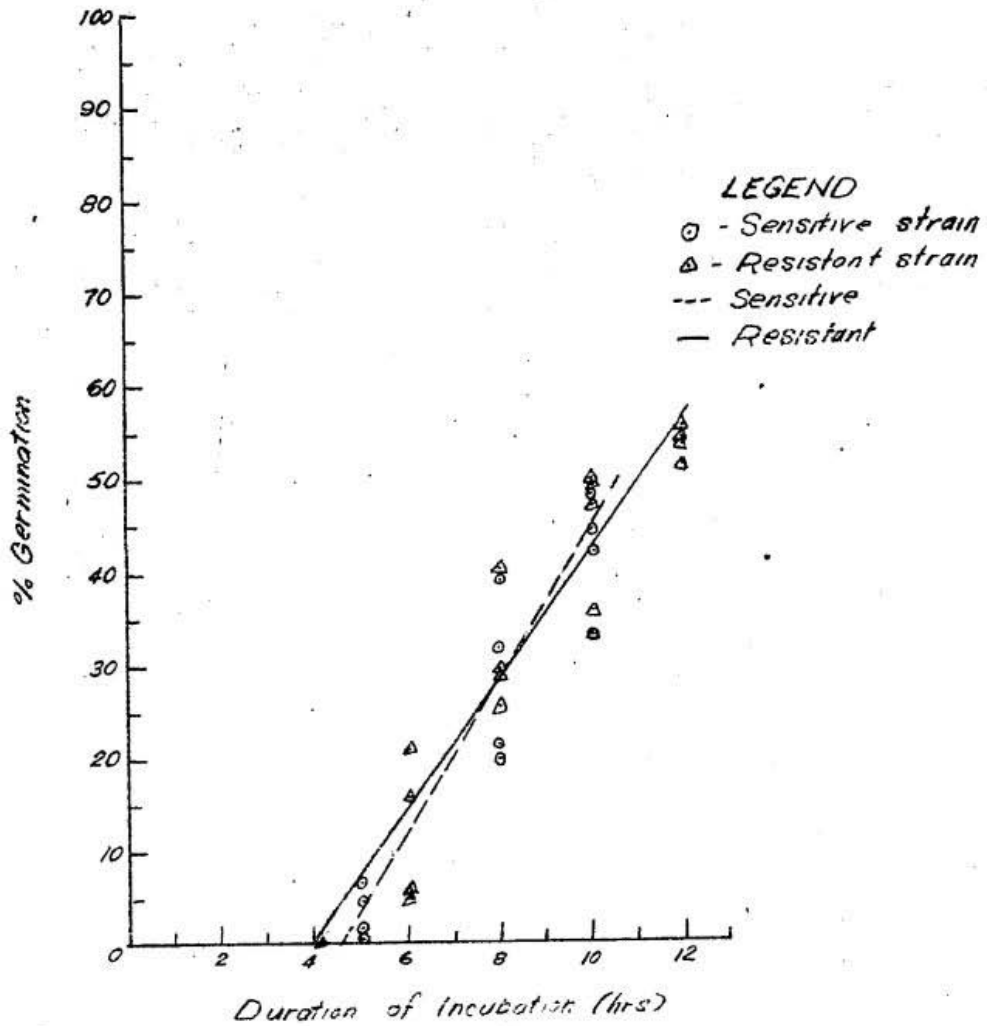


Fig. 8 Graphical Representation of Data in Table 4 (Sensitive and Resistant Strains on Complete Medium containing 800 ppm Proflavine).
N.B. One point may represent two or more identical readings - see Table 4.

TABLE 5

SENSITIVE STRAIN ON COMPLETE MEDIUM
CONTAINING 2000 ppm PROFLAVINE

GROWTH CHAMBER	# 1	# 2	# 3	# 4
RELATIVE NUMBER OF SINGLE SPORES	Many	Many	Many	Many
RELATIVE NUMBER OF GROUPS	Moderate	Moderate	Moderate	Few
DURATION OF INCUBATION (Hrs.)	PERCENT GERMINATION			
4	0	0	0	0
6	0.5	1.0	4.0	4.0
8	2.5	6.5	13.5	11.5
10	2.5	9.0	19.0	11.0
12	7.0	9.5	15.5	16.0

SENSITIVE STRAIN ON COMPLETE MEDIUM
CONTAINING 2000 ppm PROFLAVINE

GROWTH CHAMBER	# 1	# 2	# 3	# 4
RELATIVE NUMBER OF SINGLE SPORES	Few and Far Apart	Few and Far Apart	Few and Far Apart	Few and Far Apart
RELATIVE NUMBER OF GROUPS	Very Few	Very Few	Very Few	Few
DURATION OF INCUBATION (Hrs.)	PERCENT GERMINATION			
4	0	0	0	0
6	0.5	0	0.5	0
8	2.5	1.5	1.5	2.5
10	7.5	5.0	9.0	13.5
12	15.0	8.0	15.5	17.0

RESISTANT STRAIN ON COMPLETE MEDIUM
CONTAINING 2000 ppm PROFLAVINE

GROWTH CHAMBER	# 1	# 2	# 3	# 4
RELATIVE NUMBER OF SINGLE SPORES	Many	Many	Many	Many
RELATIVE NUMBER OF GROUPS	Moderate	Few	Moderate	Moderate
DURATION OF INCUBATION (Hrs.)	PERCENT GERMINATION			
4	0	0	0.5	0
6	11.5	8.0	8.0	14.5
8	51.5	34.0	27.0	31.0
10	45.0†	38.0	36.0	43.5
12	53.0	42.0	41.5	37.0†

† The disparity in per cent germination in the same growth chamber may be due to human error in counting, or spores may be germinating faster in some areas than in others. In #4 at 12 hours, some areas were so densely populated that they had to be passed over. This meant omitting many single germinating spores.

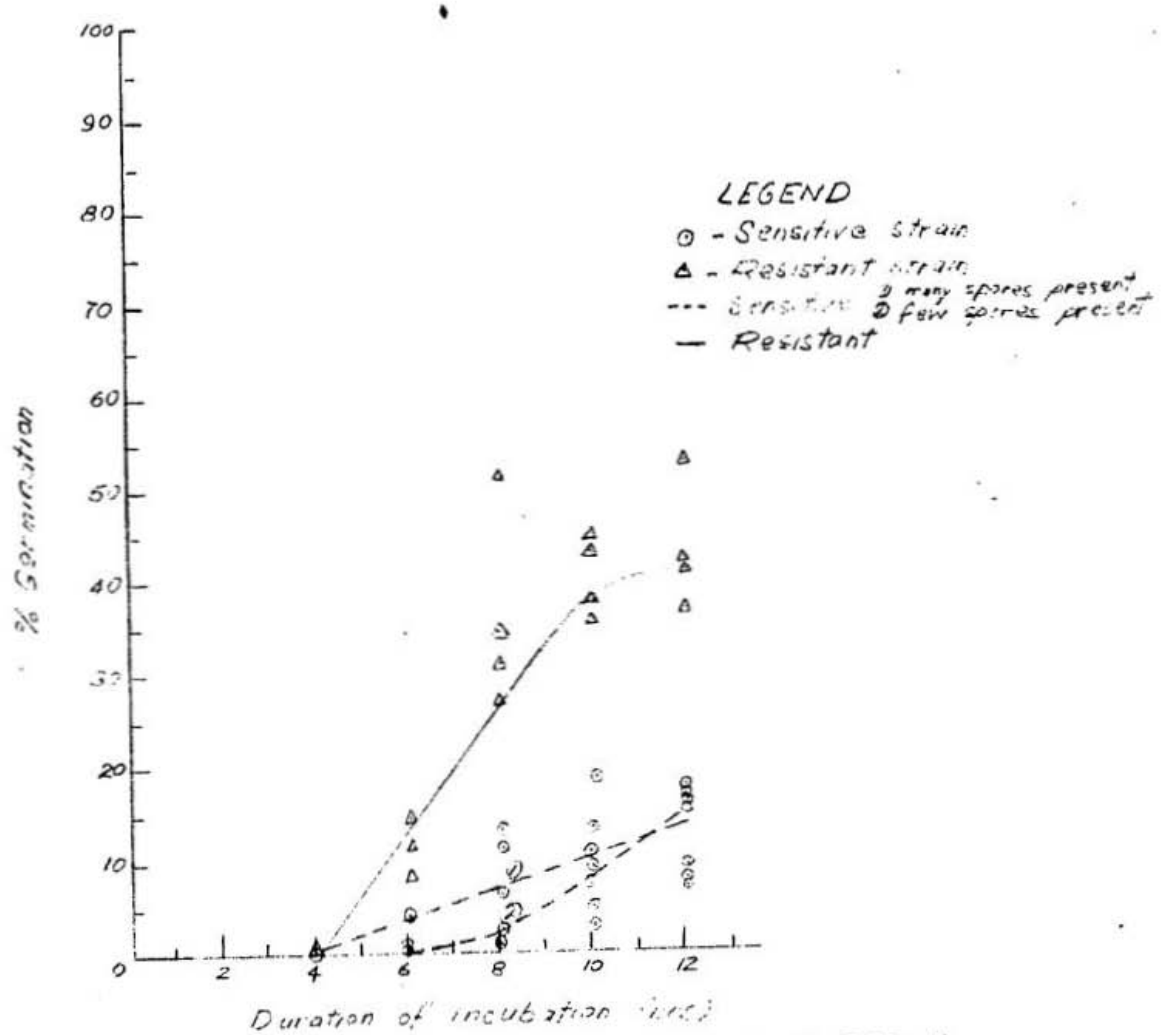


Fig. 9 Graphical Representation of Data in Table 5 (Sensitive and Resistant Strains on Complete Medium containing 2000 ppm Proflavine). N.B. One point may represent two or more identical readings - see Table 5.

hours, then starts to flatten. The sensitive curve (where many spores are present) rises linearly. The resistant strain had a much higher rate of germination and per cent germination than had the sensitive strain. The graph (Fig. 9) shows the germination rate of the sensitive strain with few spores present. This is added for the sake of comparison.

In all the chambers containing the resistant strain, there were many single spores and only one chamber had few groups. All the readings were approximately the same up to 8 hours, when hyphae started masking single spores, and the measurements were not so reliable.

Comparison of the data above:

Fig. 10 is a summary of the above results in the form of a histogram. Growth of the sensitive and resistant strains on drug-free medium has been included for the purpose of comparison. As can be seen little difference exists between the rate of germination of sensitive and resistant spores on concentrations of proflavine up to 800 ppm. However, it is significant that on drug-free medium, the resistant strain had fewer spores germinating than had the sensitive. The most significant results are those found with 2000 ppm. At lower concentrations, it appeared that the untrained strain was almost as resistant as the trained strain. However, on 2000 ppm, the resistance of the untrained strain dropped markedly. This indicates that training affects only the germination of the spores on extremely high concentrations of the drug.

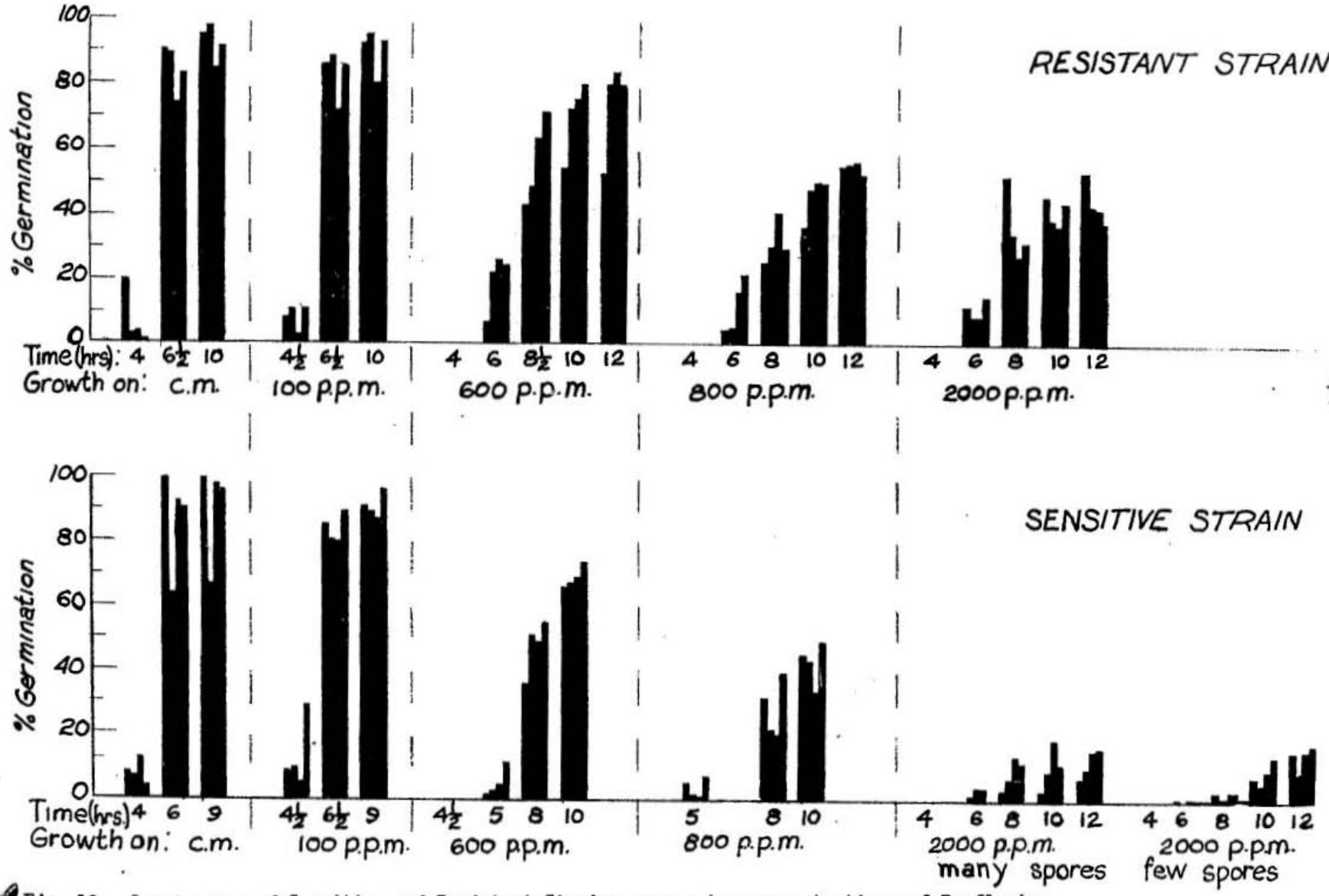


Fig. 10 Germination of Sensitive and Resistant Strains on varying concentrations of Proflavine.

In those growth chambers containing 2000 ppm proflavine, and in which spores were plentiful, both the sensitive and resistant strains started to germinate at approximately the same time (after about 4 hours incubation); this was about two hours earlier than germination began in those chambers containing the same drug concentration, but much fewer sensitive spores.

Per cent germination became gradually lower in both strains, as the drug concentration increased, until at 2000 ppm, the per cent germination for the sensitive strain at 12 hours was approximately 13% of what it had been on drug-free complete medium at 9 hours (ignoring the lowest per cent on this medium - i.e. 67%, as being unrepresentative). The per cent germination for the resistant strain at 12 hours was approximately 47% of what it had been on drug-free complete medium at 10 hours.

Even though the resistant and sensitive conidia were inoculated from four different colonies to drug-free complete medium and complete medium containing 100 ppm proflavine, approximately the same germination rate and per cent germination were obtained within each group of chambers.

Determination of size of conidia on germination

In the experiments reported below, the detailed measurements of the conidia in the preliminary swelling stage were made. Since it was found impossible to measure

the conidia until approximately one hour after incubation had elapsed, because up to this time the spores were still migrating in the film of liquid in which they had been inoculated, spores were inoculated to 2% Agar, to ensure that no swelling could take place, as it would do on complete medium, during the one hour's wait. In this manner, the size of the conidia before swelling could be measured. It was found, from the measurement of 150 conidia, that the sensitive strain had an average conidial diameter of 3.2 μ. The range was 2.7 to 3.8 μ, excluding two spores which were 4.1 μ and one which was 4.4 μ in diameter. These three spores were probably diploid. Pontecorvo (8) noted that the mean diameter of haploid conidia of the wild type of this mold was 3.1 μ (85 conidia measured) and that of the mutant y (yellow conidia) was 3.2 μ (89 conidia measured), although the range of sizes was 3.0 to 3.5 μ. When 150 resistant spores (taken from colonies on drug-free complete medium inoculated with spores from 400 ppm proflavine) were measured on 2% Agar, it was found that the average diameter was 3.1 μ.

When measurements were made of conidia on the drug-free or drug-containing complete medium, in growth chambers, two hundred (200) spores were measured. As spores germinated, they were merely counted and not measured.

Tables 6 - 11 below show the observations of swelling

SPORE SWELLING AND GERMINATION

DURATION OF INCUBATION		GERMINATING SPORES		NON-GERMINATING SPORES Diameter in μ					
Hrs.	Min.	PERCENT	NUMBER	3.7 -4.4	4.7 -5.2	5.5 -6.8	7.1 -8.2	8.5 -11.0	NUMBER MEASURED

TABLE NO.6 SENSITIVE STRAIN ON DRUG FREE COMPLETE MEDIUM

1	20	0	0	135	15	0	0	0	200
3	5	0	0	116	60	24	0	0	200
4	50	0.5	1	47	35	116	1	0	199
7	20	65.0	130	11	5	45	9	0	70
8	20	81.0	162	10	5	18	5	0	38
9	20	85.5	171	10	1	13	5	0	29
0	34	0	0	177	22	1	0	0	200
2	35	0	0	96	65	39	0	0	200
4	0	0	0	34	56	109	1	0	200
5	0	26.0	52	19	17	103	9	0	143
6	0	62.0	124	10	4	48	14	0	76
7	0	87.5	175	8	3	13	1	0	25
8	0	91.5	183	15	0	2	0	0	17
9	0	94.0	188	8	2	2	0	0	12

TABLE NO.7 RESISTANT STRAIN ON DRUG FREE COMPLETE MEDIUM

0	41	0	0	185	15	0	0	0	200
2	21	0	0	178	20	2	0	0	200
4	9	0	0	149	34	16	1	0	200
6	0	0	0	106	34	59	1	0	200
8	0	0.5	1	92	25	70	12	0	199
10	1	8.5	17	79	14	67	19	4	183
11	30	26.0	52	65	9	44	29	1	148
11	45	12.5	25	108	21	23	16	7	175
13	45	18.0	36	132	10	14	5	3	164
15	22	20.0	40	126	21	4	2	7	160
16	48	25.5	51	120	8	9	7	5	149
20	35	22.5	45	121	19	10	1	4	155
23	0	28.5	57	134	2	3	1	2	143†

† One (1) spore 13.4 μ

SPORE SWELLING AND GERMINATION

DURATION OF INCUBATION Hrs.	Min.	GERMINATING SPORES PERCENT	GERMINATING SPORES NUMBER	NON-GERMINATING SPORES Diameter in μ 2.7 -4.4	4.7 -5.2	5.5 -6.8	7.1 -8.2	8.5 -11.0	NUMBER MEASURED
0	59	0	0	127	70	2	0	0	200
3	33	0	0	49	59	92	0	0	200
4	52	1.0	2	31	26	126	15	0	198
6	21	60.5	121	18	11	37	13	0	79
7	33	79.0	158	21	7	13	1	0	42
9	15	86.0	172	18	6	4	0	0	28
10	17	92.0	184	11	2	2	1	0	16
1	1	0	0	109	87	4	0	0	200
3	44	0.5	1	17	36	134	12	0	199
4	57	26.5	53	22	13	86	26	0	147
6	21	78.0	156	20	5	17	2	0	44
7	36	88.5	177	14	3	5	1	0	23
9	29	96.5	193	5	0	2	0	0	7

TABLE NO. 8 SENSITIVE STRAIN ON COMPLETE MEDIUM CONTAINING 100 ppm PROFLAVINE

TABLE NO. 9 RESISTANT STRAIN ON COMPLETE MEDIUM CONTAINING 100 ppm PROFLAVINE

1	11	0	0	160	40	0	0	0	200
3	7	0	0	103	50	47	0	0	200
4	52	0	0	58	35	106	1	0	200
6	20	6.0	12	34	12	113	28	1	188
7	33	46.5	93	36	8	39	20	2	107
9	14	76.5	153	33	0	12	2	0	47
10	17	75.5	151	43	9	6	1	0	49
1	[illegible]	0	0	137	38	3	[illegible]	0	200
3	11	[illegible]	0	61	92	47	0	0	200
4	52	0	0	29	36	135	4	0	200
6	20	11.0	22	20	5	109	43	1	178
7	33	59.0	118	25	6	25	23	3	82
9	14	86.5	173	13	0	3	0	1	27
10	17	86.5	173	24	1	2	0	0	27

* One (1) spore 8.5 μ

SPORE SWELLING AND GERMINATION

DURATION OF INCUBATION Hrs./Min.	GERMINATING SPORES		NON-GERMINATING SPORES Diameter in μ					
	PERCENT	NUMBER	2.7 -4.4	4.7 -5.2	5.5 -6.8	7.1 -8.2	8.5 -11.0	NUMBER EXAMINED
1	5	0	0	101	90	1	[illegible]	0	200
3	50	0	0	29	76	95	0	0	200
6	20	43.5	87	12	27	71	3	0	113
7	36	55.0	110	14	30	50	6	0	90
9	44	85.5	171	7	11	3	3	0	29
10	46	90.5	181	4	4	10	0	1	19
11	36	90.0	180	10	4	5	1	0	20

TABLE NO. 10 SENSITIVE STRAIN ON COMPLETE MEDIUM CONTAINING 600 ppm PROFLAVINE

1	2	0	0	179	21	0	0	0	200
3	43	0	0	97	77	26	0	0	200
6	20	13.5	27	92	34	84	3	0	173
7	36	27.5	55	56	25	53	11	[illegible]	145
9	37	61.5	123	4[illegible]	16	19	2	0	77
10	46	66.0	132	3[illegible]	14	14	2	0	68
11	36	73.5	147	39	6	7	1	0	52
1	5	0	0	172	26	[illegible]	0	0	200
3	48	0	0	89	76	35	0	0	200
6	21	7.0	14	57	51	77	1	[illegible]	136
7	36	22.5	45	45	[illegible]	63	13	0	155
9	34	44.5	89	46	16	39	2	0	103
10	46	55.5	111	42	20	23	4	0	89
11	36	63.5	127	37	13	20	2	0	73

TABLE NO. 11 RESISTANT STRAIN ON COMPLETE MEDIUM CONTAINING 600 ppm PROFLAVINE

and production of a germ tube at intervals following inoculation on the media designated. The results are taken from two growth chambers in each case, except that of the original sensitive strain on 600 ppm proflavine.

In every case, there was a large proportion of conidia in the size range between 5.5 and 6.8 μ, just before the production of germ tubes was observed. However, since conidia of diameter 7.1 to 8.2 μ did appear in every growth chamber, and generally there were none of a greater diameter, it is reasonable to assume that spores must have swollen to this size just prior to production of the germ tube, especially since their number decreased markedly as maximum germination per cent was attained. Therefore, those of diameter 6.5 to 6.8 μ must have continued to swell and replaced those which had germinated. In Tables 6-11 , measurements are grouped according to an arbitrary system. The size ranges within these groups were selected following the conversion to microns of measurements made originally on an arbitrary microscopic scale.

Size prior to germination is therefore between 7.1 μ and 8.2 μ, in all cases. The lower the germination per cent, at the end of the set of counts for any growth chamber, the higher the number still remaining in the size group between 2.7 μ and 4.4 μ.

Tables 6-11 show the sizes of spores found in growth

chambers containing drug-free complete medium, and complete medium containing 100 and 600 ppm proflavine. Since measurement on the micrometer scale could be brought only approximately to 2 decimal places, in the conversion of this measurement to microns, only one decimal place was justified, and this accounts for the break seen in the range of sizes in the Tables.

In the two growth chambers containing drug-fee complete medium and sensitive spores, after an incubation period of approximately 9 hours, and when the germination per cent was high, the number of conidia in the size group 2.7 - 4.4 μ was 8 and 10. The number in this size group in the two growth chambers containing the resistant spores on drug-free complete medium was much larger (65 and 134) at 11½ hours and 23 hours incubation respectively, when the germination per cent was low. This indicates that the majority of those spores which did not germinate, did not swell beyond 4.4 microns. There were a number of resistant spores in the second growth chamber, which had swollen to a diameter between 9.0 and 11.0 μ, and one which had swollen to 13.4 μ. In spite of the fact that they had been in the growth medium for 23 hours, these had not germinated.

The per cent of resistant spores which germinated on 100 ppm proflavine was lower than that of the sensitive spores on the same medium. The drug had no effect on the

size of the conidia at which the germ tube was observed to appear. The number of spores of diameter between 2.7 and 4.4 μ was almost equal to the total number which failed to germinate. There were no excessively large conidia present.

On 600 ppm proflavine, of those conidia which had not produced germ tubes after 11 hours, about fifty percent were in the size range 2.7 to 4.4 μ. The development of spores of both untrained and trained strains followed the usual course, and a germ tube was again produced when the conidia had reached a size between 7.1 and 8.2 μ.

Size necessary for complete germination was not affected by the proflavine, since the spores, whether resistant or sensitive, and whether on complete medium alone or on drug-containing complete medium, produced a germ tube when they reached a size between 7.1 and 8.2 μ. Exceptions to this were occasionally observed, in conidia of the trained strain, which sometimes reached a greater diameter without producing a germ tube.

Germination of the Conidia of the Trained and Untrained Strains on 2000 ppm Proflavine.

In a separate experiment, an attempt was made to determine the effect of a high concentration (2000 ppm) of proflavine, on the germination of conidia taken from trained

and untrained strains. Approximately 5×10^4 conidia of each strain were inoculated to duplicate sets of growth chambers containing complete medium plus 2000 ppm proflavine. One hundred (100) ungerminated spores of each were observed at intervals following inoculation, and measurements were made as described earlier. As germinating spores were observed, these were not included in the total count of 100. The results of these observations are given in Table 12

It will be seen that on this high concentration of proflavine, a high proportion of the ungerminated spores of both strains remained in the size group up to 4.4 μ, and of the remainder, a very small number of the spores exceeded 7.1 μ in the ungerminated state. As shown in the Table, many of the spores which attained 7.1 μ were observed to have altered slightly in shape, indicating the early phase of emergence of a germ tube. At approximately 9 hours, nearly 100% of those conidia which had not produced a germ tube, remained in the size group up to 4.4 μ. Of the conidia which had, at approximately 9 hours, increased beyond this size, many gave the impression that a germ tube would soon be produced.

The hyphae produced by conidia of both strains, which had successfully germinated, were observed to be more sparse and shorter than is usual on drug-free complete medium. No sporulation was observed in any of the growth chambers up to 54 hours.

TABLE NO. 12

DIAMETER OF NON-GERMINATING CONIDIA ON 2000 ppm PROFLAVINE

DURATION OF INCUBATION (Hours)	NON-GERMINATING SPORES					
	Diameter in μ					
	Up to 4.4	4.7-5.2	5.5-6.8	7.1-8.2	8.5-11.0	Number measured
SENSITIVE STRAIN ON COMPLETE MEDIUM CONTAINING 2000 ppm PROFLAVINE						
2	87	13	0	0	0	100
2½	87	13	0	0	0	100
4	82	12	6	0	0	100
4½	80	11	9	0	0	100
6	75	9	14+2*	0	0	100
6½	83	6	8+1*	2	0	100
9½	94	0	2	2+2*	0	100
9½	97	1	2	0	0	100
RESISTANT STRAIN ON COMPLETE MEDIUM CONTAINING 2000 ppm PROFLAVINE						
2	94	6	0	0	0	100
2½	91	9	0	0	0	100
4	87	8	5	0	0	100
4½	83	8	9	0	0	100
6	90	3	6	1*	0	100
6½	86	3	11	0	0	100
9	95	0	1+2*	2*	0	100
9½	95	2	2+1*	0	0	100

*Because of changes in shape of conidium at this stage, it was suspected that the germ tube had just begun to emerge.

CHAPTER II

THE EFFECT OF PHENYLMERCURIC ACETATE ON THE GERMINATION OF SENSITIVE AND RESISTANT STRAINS OF THE MUTANT

Concentrations of phenylmercuric acetate of 0.20 ppm were toxic to the untrained strain of the mutant studied here. On subculturing to gradually increasing concentrations of this inhibitor, growth could be obtained on as high as 1.10 ppm phenylmercuric acetate, with an inoculum estimated at 10^6 conidia (Curren, T.; 10). It was found by Curren that resistance was lost at two points in the training process: spores taken from a colony on 0.45 ppm could not grow on concentrations of the inhibitor higher than 0.30 ppm; and spores from a colony on 0.60 ppm would not grow on a concentration of phenylmercuric acetate above 0.40 ppm. On reducing the concentration of the inhibitor, however, growth of the organism occurred, and the training process could be continued.

The trained strain used in the experiments reported here was that obtained by Curren on 0.50 ppm phenylmercuric acetate. It was not continuously subcultured to 0.50 ppm. Whenever growth chambers were to be inoculated with this trained strain, conidia were taken from a single colony on the 0.50 ppm phenylmercuric acetate plate and plated to drug-free complete medium and incubated at 37° for 37 hours.

Conidia were then taken from a single colony on the drug-free plate and inoculated to the growth chambers. When plates were inoculated, the conidia were taken directly from the 0.50 ppm phenylmercuric acetate plate.

Level of Resistance of the Sensitive Strain to Phenylmercuric Acetate IN GROWTH CHAMBERS:

Growth chambers containing 0.50 ppm phenylmercuric acetate were inoculated with spores of the mutant strain which had been shown to be sensitive to this concentration. The precise manner in which the drug prevented germination could thus be studied microscopically by examining its effect on the process of germination. No account was taken of the first stage in germination, that is, the swelling of the spores, but only of the emergence of the germ tube.

In preliminary experiments the rate of germination was found to be affected by clumping of spores. Therefore, only general conclusions can be drawn from data which did not take into account that germination of isolated spores in groups should be considered differently.

Two of the observations made which led to the conclusion that clumping of spores increases the rate of germination may be given. In one instance, there was 6% germination among isolated single spores (i.e. where no contact between spores was observed) after 11 hours incubation, but 90% among spores in

groups. In another, 88% of the single spores had completed the germination process after 17½ hours incubation; most in groups showed close to 100% germination.

Perhaps as a consequence of the presence or absence of many groups, the time at which germination commenced could not be pinpointed.

Germ tubes appear at some period between 5½ and 8 hours after commencement of incubation. This at least indicates that the completion of germination is retarded by at least 1½ hours in the presence of phenylmercuric acetate, since on drug-free complete medium, germ tubes emerge after about 4 hours incubation.

Sporulation was ordinarily not accomplished, even though the incubation period extended to 123 hours. Sporulation did occur once, and this was after an incubation period of 51 hours.

In preliminary experiments it was observed that dark areas had appeared in the hyphae of the sensitive strain grown in the presence of 0.50 ppm phenylmercuric acetate. Three (3) growth chambers containing 0.50 ppm phenylmercuric acetate were inoculated with the sensitive strain. It was observed in all three that deposits were seen in the hyphae after 25 hours incubation. No deposits had appeared after 17½ hours incubation, but they were evident after 22 hours incubation. Germ tubes had appeared after 8 hours incubation, but no deposits were observed in the germ tubes or hyphae

after 8 or 11 hours incubation. The deposits were fairly regularly spaced along the length of the hyphae. Figure 11 is a reproduction of two photomicrographs taken of sensitive spores which had germinated on 0.50 ppm phenylmercuric acetate. The deposits can be seen. For comparison, photomicrographs of isolated single sensitive spores and a group of sensitive spores germinating on drug-free complete medium are given in Figure 12.

After varying periods of incubation (from 25 to 40 hours), growth of the hyphae stopped or was greatly retarded, and there was never the great mass of hyphal growth which is seen on drug-free complete medium. The hyphae eventually (after approximately 50 hours incubation) looked like dead, dry, branches of trees. The tips of the hyphae could be clearly seen and were thin, dessicated and wizened.

Determination of Level of Resistance of Strain trained to grow on 0.50 ppm phenylmercuric acetate.

ON PETRI PLATES: Colony formation

The sensitive strain had been trained by a laboratory colleague (unpublished) to grow on 0.50 ppm phenylmercuric acetate, a concentration normally toxic to this organism. To test the level of resistance of this trained strain, a small number of spores were taken from colonies grown in the presence of 0.50 ppm phenylmercuric acetate, and inoculated to a drug-free complete medium plate and to

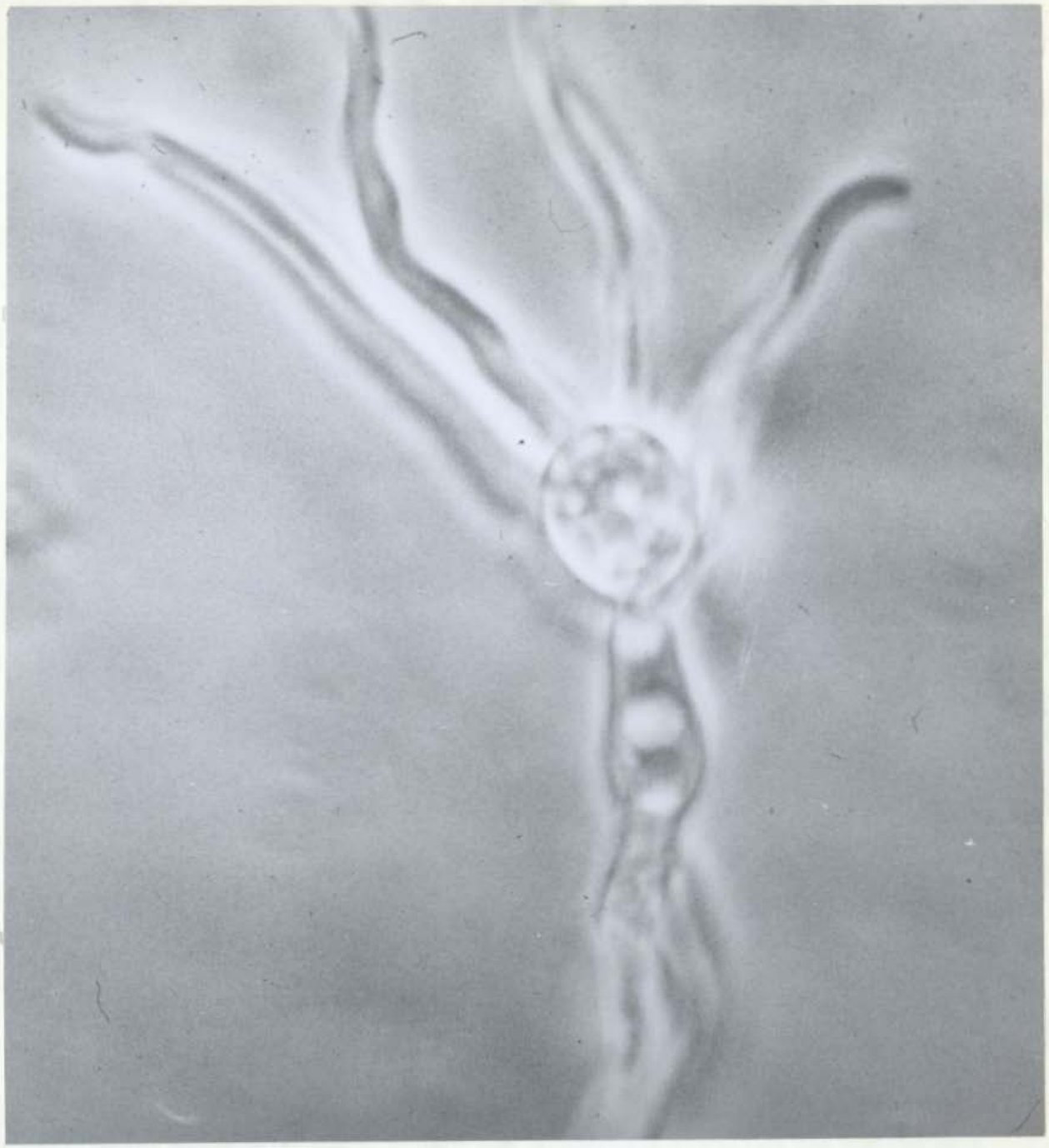


Fig. 11 (a) Photomicrograph of isolated sensitive spore on 0.50 ppm phenylmercuric acetate, after approximately 26 hours incubation (x 4000). Note the deposits in the hypha below the spore.

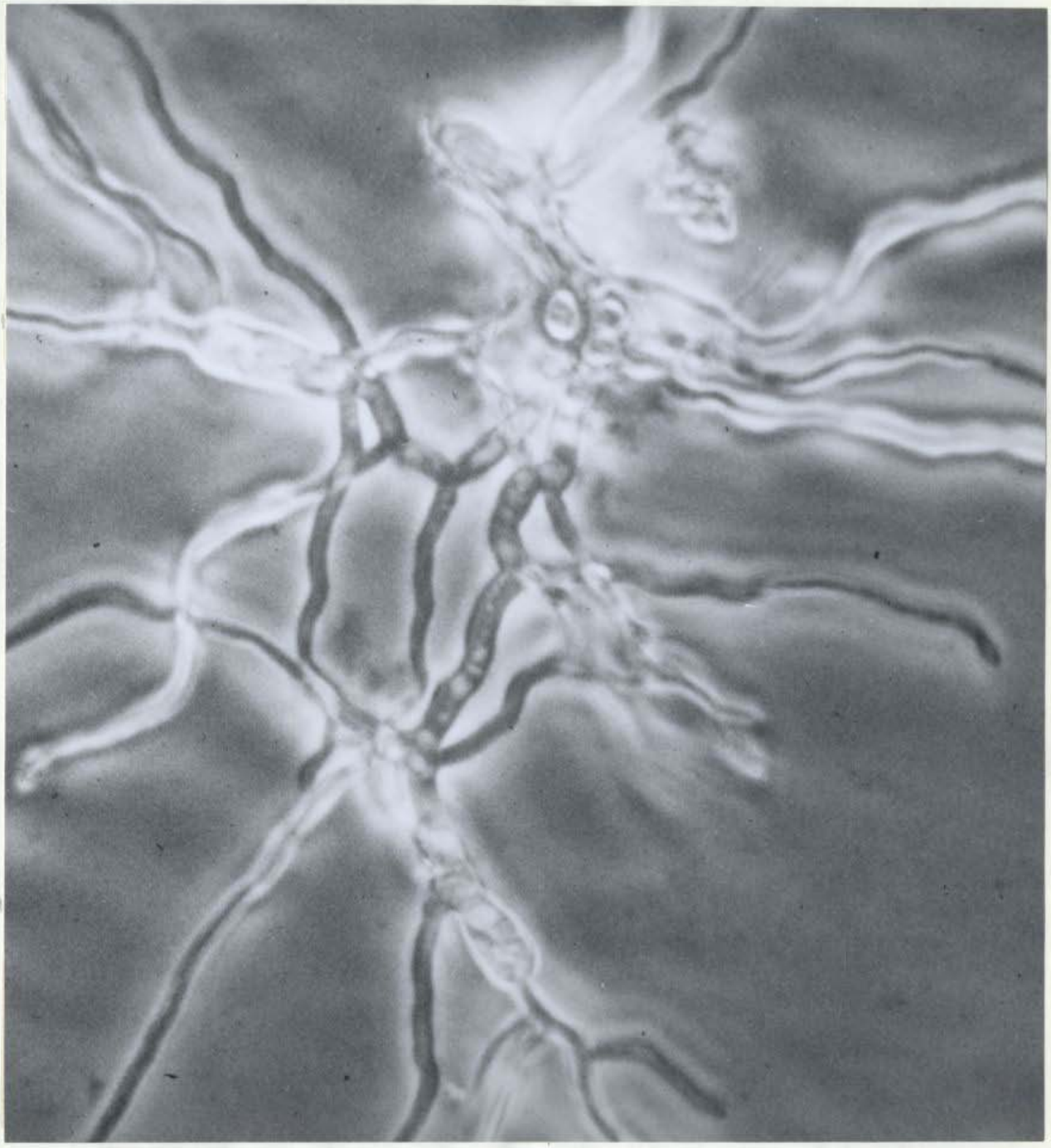


Fig. 11 (b) Photomicrograph of group of sensitive spores on 0.50 ppm phenylmercuric acetate, after approximately 29 hours incubation (x1100). Note the deposits in the hyphae.

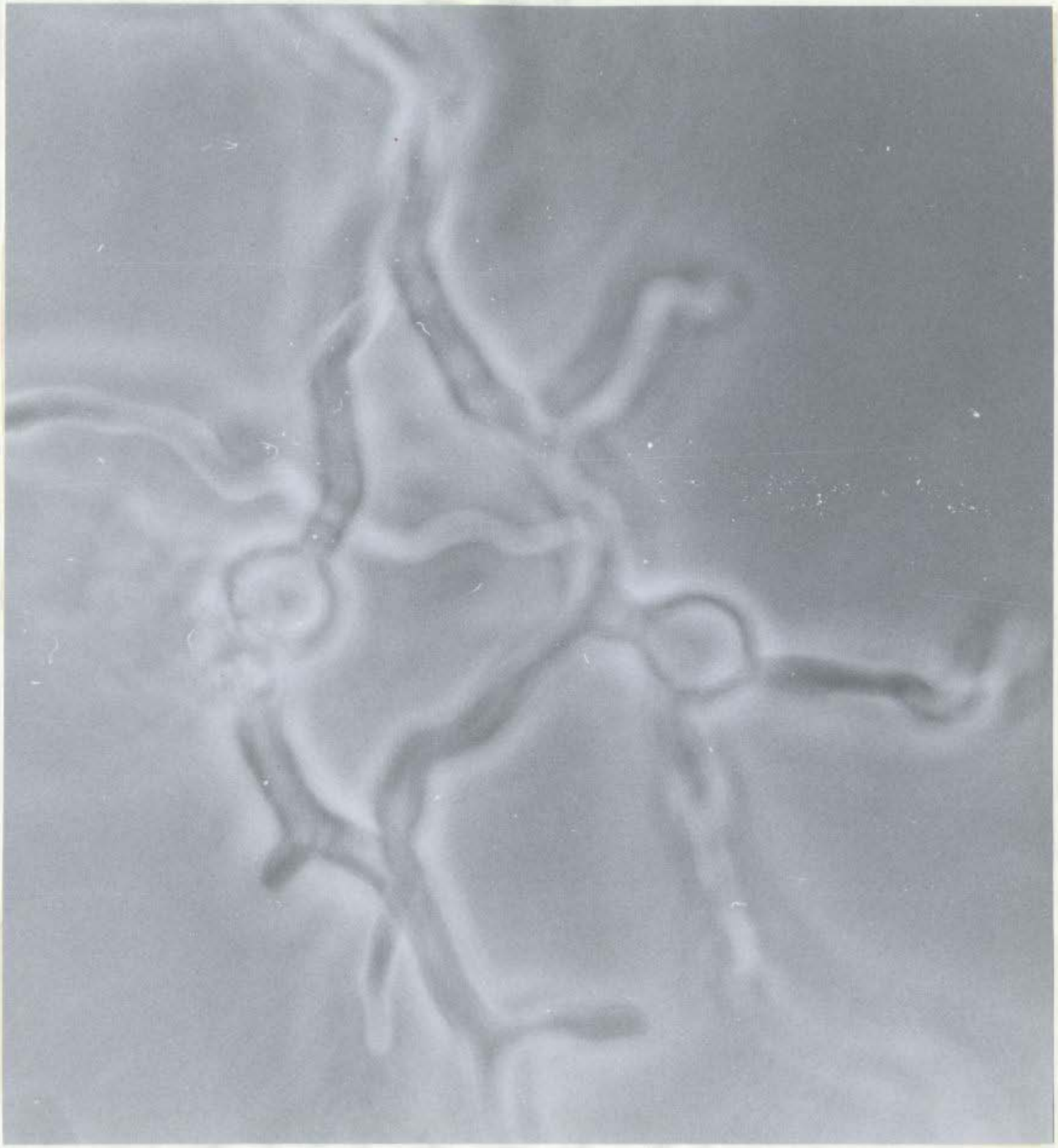


Fig. 12 (a) Photomicrograph of sensitive isolated single spores on drug-free complete medium, after approximately 10½ hours incubation. (x 2000)

Fig. 12 (b) Photomicrograph of group of sensitive spores on drug-free complete medium, after approximately 22 hours incubation. (x 550)

plates with complete medium containing 0.40, 0.45, 0.50, 0.55, and 0.60 ppm drug. Although abundant growth was produced on the drug-free plate, no growth was produced on any drug plate, even after 110 hours incubation. 100 spores, taken from the drug-free plate of this group, were tested on the same range of concentrations. Drug plates produced no growth, even after 65 hours incubation. Good growth was obtained on the drug-free plate.

To determine whether the size of inoculum might be influencing growth, a heavy inoculum (approximately 10^6 conidia) and a dilute inoculum (approximately 10^3 conidia) were inoculated to the same range of drug concentrations: drug-free complete medium and complete medium containing 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55 and 0.60 ppm.

In both instances, growth occurred on the drug-free plates after 22 hours incubation. At this time there was growth on the heavily inoculated plates containing 0.25, 0.30 and 0.60 ppm phenylmercuric acetate. There was no growth on the drug plates containing dilute inoculum.

Other readings were made after about 32 hours and 51 hours incubation. At 32 hours, complete sporulation had occurred on the abundant growth present on the heavily inoculated drug-free plate and sporulation had started on the 0.25 ppm plate. There was about 75% sporulation on the

good growth obtained on the drug-free plate containing dilute inoculum. No other sporulation was evident on the plates of either group.

At this time, there was light growth on 0.30 ppm, 25 colonies on 0.35 ppm, 1 colony on 0.40 ppm, and 40 colonies on 0.60 ppm, where heavy inoculum had been used. Where light inoculum had been added, there were 15 small colonies on 0.25 ppm and 1 small colony on 0.30 ppm.

On the heavily inoculated plates, after 51 hours incubation, the number of colonies on 0.25, 0.30, 0.35, 0.40, and 0.60 ppm had increased and sporulation had started on all but the 0.40 ppm plate.

At the same time, on the plates with dilute inoculum, there was still no growth on the plates containing drug in concentrations ranging from 0.35 to 0.60 ppm. The number of colonies on the other drug plates (0.25 and 0.30 ppm) had increased. Sporulation had begun on both these plates.

Thus, only on those plates containing heavy inocula was there any growth on concentrations higher than 0.30 ppm phenylmercuric acetate. It may be observed that the successful growth of the strain on 0.60 ppm may well have been an artifact, due to some technical error in the preparation of the media. Since the conidia inoculated to all plates were taken from the same colony, it is unlikely that the appearance of 40 colonies on 0.60 ppm phenylmercuric

acetate indicates the selection of mutants.

IN GROWTH CHAMBERS: (containing 0.50 ppm phenylmercuric acetate)

We have seen that heavy inoculum aided germination of spores from the trained strain on drug plates. Growth chambers were therefore prepared in order to observe the effect of this concentration of the drug upon the various phases of germination.

In three growth chambers studied, germ tubes began to appear after three different periods of incubation, i.e. 6, 9 and 11 hours. In the second of these, the clumping of spores increased the germination rate. Thus, at 9 hours, spores within groups had started to produce germ tubes and at 11 hours, 16% of single isolated spores had completed germination, while 30 to 88% of spores in groups had completely germinated.

There was no rate of germination common to all the chambers. Two hours after the appearance of germ tubes, in one chamber 40% of single spores had completely germinated, and in another, only 16% had completed the process.

Sporulation occurred in all three growth chambers. In one case, it had begun after 47½ hours incubation. In another case, it occurred at 24½ hours. However, in the chamber in which germ tubes had not been produced until after 11 hours incubation, the sporulation occurred after

approximately 50 hours incubation, and only in an area of dense growth which had initiated from the periphery.

At first only a few conidial heads were seen, each with chains of 3 or 4 conidia. After further incubation, there were many large conidial heads seen. Those conidia which had apparently matured were approximately 2.7 to 3.0 μ in diameter. The conidiophores were shorter than normal. Although not enough conidiophores were measured to allow calculation of a statistically satisfactory average of the lengths, it was seen that one conidiophore was 82.2 μ in length and that none appeared to be longer than this. Pontecorvo (8) states that the conidiophore of this mold is 100 μ in length.

Again the hyphae contained what has already been described as deposits. Three growth chambers were prepared containing medium containing 0.50 ppm phenylmercuric acetate, and were inoculated from colonies which had been grown separately on high concentrations of the inhibitor. In 2 of the 3 growth chambers where conidia had been taken from plates containing 0.50 ppm phenylmercuric acetate, germ tubes were observed after approximately 7 hours incubation, and deposits were noted two hours after the initial appearance of the germ tubes. In the third growth chamber, which was inoculated with spores taken from a colony grown on 0.45 ppm of the same drug, germ tubes did not appear until after approximately 20 hours, but hyphae contained deposits

some 6 hours after the first appearance of the germ tubes. In this latter case, only a small percentage of the inoculated conidia eventually completed germination. In one instance the deposits caused the walls of the hyphae to bulge out and in at least one hypha, the deposits appeared to be breaking through the hyphal wall. After various periods of incubation (23 hours or more) the hyphae became dessicated, gnarled and woody-looking. They were evidently not being permitted to ramify and/or lengthen, as those of the untrained strain would on drug-free medium, since it was easy to distinguish the conidial heads, which are usually masked by the hyphae when the fungus is grown on complete medium alone.

In the growth chamber in which commencement of germination had been delayed the longest, it was seen that there were germinating spores which were misshaped (prune-like) and some were grossly enlarged. Some spores which had not germinated were also greatly enlarged (up to 12.3 μ in diameter). One spore was 11.0 μ in diameter and was filled with deposits. In this same chamber spores were puckered and dried after 29 hours incubation.

Further Training of the Resistant Strain in Growth Chambers:

It has earlier been remarked that in most cases, growth of the resistant strain in petri plates was inhibited by concentrations higher than 0.50 ppm phenylmercuric acetate.

It has also been noted that resistant spores were normally more successful in growth chambers than on petri plates. In an attempt to further pursue this observation, spores trained to grow in the presence of 0.50 ppm phenylmercuric acetate on petri plates were inoculated to a growth chamber containing complete medium with 0.60 ppm phenylmercuric acetate. Over a period of 54 hours, the inoculated conidia were studied and measured. It was observed that no germination occurred prior to 26 hours from the time of inoculation, and that of 150 isolated spores measured, the average diameter was 3.1 μ. No conidium attained a diameter greater than 4.1 μ. At 26 hours, a small colony appeared at the site of a group of spores. This single colony produced conidiophores prior to 36 hours, and what appeared to be normal conidial heads at 50 hours. No other germination was observed, and 150 ungerminated spores measured retained an average diameter of approximately 3.0 μ.

In a similar experiment, it was found that conidia of the trained strain referred to above, failed to increase markedly in diameter over a period of 78 hours, in a growth chamber containing complete medium and 1.00 ppm phenylmercuric acetate. The average of 150 isolated conidia measured at intervals during the experiment was 3.2 μ. No conidium exceeded a diameter of 4.4 μ. No germination was observed. This demonstrated that 1.00 ppm phenylmercuric acetate could completely inhibit all phases of germination in the strain previously trained to grow on petri plates containing a

concentration of 0.50 ppm phenylmercuric acetate.

In an attempt to determine if the single colony of the trained strain, which had appeared on 0.60 ppm phenylmercuric acetate, was markedly more resistant than the parental strain, conidia were transferred from 0.60 ppm phenylmercuric acetate to a growth chamber containing complete medium and 0.80 ppm phenylmercuric acetate. The conidia were studied microscopically at intervals over a period of 31 hours, and it was found that at 17 hours, germ tubes had been produced in a number of groups of conidia. At this point, no isolated conidia had begun to germinate. At 24 hours, the average diameter of 100 spores in groups, not yet showing a visible germ tube, was 7.3 μ, while the average of 100 isolated spores at the same stage, was 3.0 μ. A small number of isolated spores had shown a visible germ tube. No sporulation was observed either in isolated spores or in groups of spores. By 31 hours, sporulation had been accomplished in both isolated conidia and groups of conidia.

From the growth chamber containing 0.80 ppm phenylmercuric acetate, conidia were removed and were inoculated to a growth chamber containing 1.00 ppm. 7 hours after inoculation, it was noted that germ tubes had appeared in a number of isolated spores, and as well among spores in groups. Approximately 4% of 208 isolated spores had produced visible germ tubes at this stage. The average

diameter of 100 spores in groups at 7 hours was 4.9 μ, while the average diameter of 100 isolated spores was 5.5 μ. At 27 hours conidial heads began to appear. At 32 hours measurement of the conidiophores showed their average length to be 22.7 μ. At 48 hours, 50 conidiophores were measured and were found to have an average length of 50.5 μ (Table 13). All conidiophores were observed to have produced chains of conidia, and appeared otherwise normal.

TABLE NO.13 Lengths of Conidiophores of the Trained Strain on 1.00 ppm Phenylmercuric Acetate (in growth chamber; after 48 hours incubation).

Length (μ)	Number of conidiophores of this length	Length (μ)	Number of conidiophores of this length
131.5	1	46.6	1
109.6	1	41.1	2
95.9	1	38.4	1
74.0	3	35.6	3
71.2	2	32.9	2
68.5	1	30.1	1
63.0	2	27.4	3
60.3	2	24.7	3
57.5	1	19.2	2
54.8	10	16.4	1
52.1	4	13.7	1
49.3	2		

Average length: 50.5 μ Standard deviation: 22.7 μ

No. of conidiophores measured: 50.

DISCUSSION

Cochrane (11) states that the three morphological events of typical spore germination in A. nidulans are: nuclear division, swelling of the spore, and emergence of the germ tube. Criteria used in the experiments presented earlier were the swelling of the spore and the emergence of the germ tube. Taking the fungi generally, he says that some or all of the nutrients necessary for germination are present in the spore ab initio. Complete independence, partial dependence, and complete dependence on the environment reflect the adequacy or availability of the reserve substances of the spore. Spores of most fungi germinate best at pH 4.5 to 6.5, with limits at about pH 3 and pH 8. There is a minimum relative humidity at which detectable germination occurs. Species of Aspergillus form spores which germinate at relative humidities near 80%. Spores of many fungi are found to germinate only in liquid water.

The enzyme systems are, on the whole, those found in many other micro-organisms. The cytochrome system (Cochrane; 11) appears to be general in the fungi. However, it is not possible to state whether the fungal cytochromes are the same as those of yeast. The cytochrome system is involved in the most common terminal pathway (i.e. the pathway by which the electrons withdrawn from the substrate are transferred

to oxygen). This pathway involves a pyridine nucleotide, a flavoprotein, and the cytochrome system in succession, that is, the flow of hydrogen (electrons) is over the pathway:

Substrate → pyridine nucleotide → flavoprotein → cytochromes → oxygen

Cochrane also states that cytochrome oxidase seems to be of universal occurrence in the fungi. Catalase is present in all fungi that have been examined for it. Catalyzing the breakdown of hydrogen peroxide, the enzyme may serve only to protect the organism against hydrogen peroxide or may have a broader function in the oxidation of organic molecules.

The organic acids of the citric acid cycle which are formed in substantial amounts by fungi include succinic, citric, fumaric, and acetic acids. That the whole cycle is present in fungi generally, is not known, but Moses (12) has shown that tricarboxylic acid cycle reactions can be carried out by the fungus Zygorhyncus moelleri.

In all experiments described earlier, the conidia constantly produced a germ tube on reaching a size of approximately 7.0 μ. A few spores larger than this were observed which had not yet completed germination by producing a germ tube. The presence of proflavine in the medium did not alter this relationship. Whether from trained or untrained strains, in the absence or presence of proflavine,

the majority of those spores which failed to germinate did not exceed a diameter of 4.4 μ. Those spores which succeeded in increasing their diameter beyond this size, usually completed the germination process. This would seem to indicate that if a spore cannot swell beyond these lower limits, it is not likely to germinate, and any agent which could keep it within these bounds could effectively stop germination.

At lower concentrations, proflavine did not demonstrate marked effect upon the swelling of conidia. At the highest concentration employed (2000 ppm), however, it has been noted that close to 100% of those spores which did not complete germination retained a size less than 4.4 μ.

Thus the first effect of proflavine observed in these experiments has been the inhibition of swelling in those spores which did not germinate. In order to swell, spores must absorb water and nutrients. Cochrane (11) relies on the generalizations of Yarwood that fungus spores with a low water content must absorb water to about a level of 70% before they can germinate, and says that this hypothesis explains the swelling of spores and would demand that all spores of low water content swell on germination. Reserve substances must also be produced. Those conidia which did not swell in the present experiments either could not absorb water or could not build up the necessary reserve of substrates, or both. The inhibitory effect of proflavine on germination

may therefore be due to either (a) its ability to decrease the permeability of the cell wall or (b) its ability to inhibit metabolism, causing death of the spore before it could absorb water and nutrients.

A second general phenomenon observed in all strains is that the number of spores present affect the initial rate of germination in the presence of the inhibitor. This is shown in Table 5, shown earlier. When many spores were present, the sensitive strain started to germinate earlier on high concentrations of proflavine than it did when few spores were present. The effect of spore numbers could not be shown with the groups of growth chambers containing drug-free complete medium and complete medium containing 100 to 800 ppm proflavine, since the relative number of spores was not noted.

Cochrane (11) states that it has been commonly observed that excessive crowding of spores on a surface results in reduced germination; the phenomenon is known in many fungi, and may be described by the general term self-inhibition. He says that the endospores of Bacillus globigii produce a substance which inhibits germination of fresh endospores of the same species. Allen determined several characteristics of the substance - possibly substances - responsible for self-inhibition of Puccinia graminis tritici uredospores. The inhibitor is a spore metabolite produced under aerobic conditions. Cochrane states (at page 414):

"This differs somewhat from the situation in Aspergillus niger and Coccomyces hiemalis, in both of which limited evidence suggests that an inhibitor is present in the spore at the time of its formation and can be removed by washing." In Neurospore crassa, crowding of conidia reduces the germination rate, but not total germination.

Thus, though self-inhibition is reported on drug-free medium, it has here been observed that on drug medium, crowding of spores increases the initial germination rate. Group-protection must be afforded by these spores. A. nidulans may produce a metabolite, like the one responsible for self-inhibition. On the other hand, perhaps the form of protection is merely the removal of the proflavine from the medium surrounding the spores, so that others are not affected by the high concentration of the drug. These others can then manage to get nutrients and water from the medium, or at least carry on until an alternate metabolic pathway has been established to supplant the one inhibited by the drug.

It has been observed that concentrations of less than 800 ppm of proflavine affect equally the germination of conidia of trained and untrained strains. It can therefore be assumed that training does not increase the ability to germinate on these concentrations. In medium containing 2000 ppm, however, it was noted that a higher proportion of the conidia of the trained strain (i.e. those strains which

had been cultured continuously on subtoxic concentrations of the inhibitor) succeeded in completing the germination process than did those of the untrained strain. This difference is especially obvious in Fig. 9.

An alternate pathway for metabolism may prove necessary for the spore to overcome the inhibitory effects of the drug. In experiments dealing mainly with bacteria, it has been found that proflavine affects enzymes adversely. James and Hinshelwood (13) showed that proflavine inhibited dehydrogenase activity and prevented growth of Bacterium lactis aerogenes. In general the proflavine concentrations needed to inhibit the enzyme activity were much higher than those which prevented growth. Training of the cells induced resistance to the action of the drug on dehydrogenase activity, as well as on growth. It was shown by Cole and Hinshelwood (14) that catalase activity in the same organism was reduced by adaptation to proflavine. Scopes and Hinshelwood (15) and Dean and Hinshelwood (16) found that a proflavine-trained strain of Bact. lactis aerogenes was unable to ferment sucrose or to form acetylmethylcarbinol from glucose, and showed some adaptation to succinate. Slonimski and Ephrussi (17) showed that proflavine causes damage to the cytochrome system of yeasts. Dean (18) thinks it feasible that training to proflavine may lead to an increase in the amount of succinic dehydrogenase to compensate for its decreased activity.

If the drug interferes with the enzyme systems in A. nidulans, some mechanism must come into play during training to permit germination of resistant spores in the presence of high concentrations of the drug. Perhaps an enzyme susceptible to inhibition by proflavine is produced to excess. Perhaps an alternative route of metabolism has to be geared to action. As a consequence, there would be a delay before germination began, similar to the pronounced "lag" in bacteria produced in the presence of inhibitors (Davies, Hinshelwood and Pryce; 19). Slight, if any, delay in the commencement of germination of A. nidulans spores was observed in most of the experiments described earlier, in which trained or untrained strains were permitted to grow in the presence of proflavine. Only where untrained spores in low numbers grew in the presence of a very high concentration (2000 ppm) of proflavine was a marked lag (2 hours) observed.

It was observed microscopically that, once a germ tube had been produced, hyphae continued to grow, although high concentrations of proflavine restricted their growth considerably. Since the hyphae derive their nuclei from the spore, they may have inherited the ability to grow on the drug. On the other hand, it may be suggested that in the process of metabolism the hyphae change the pH of the surrounding medium. If the pH of the medium is sufficiently acidified, the action of the proflavine is reduced. Thornley et al (20) showed this with the organism Bact. lactis aerogenes.

If the pH of the growing culture was kept neutral, growth ceased on addition of proflavine. But if the culture was acidified (pH 4.9) growth continued, even on addition of concentrations of proflavine which had originally stopped growth. Albert (21) considered pH effect further. He pointed out that it is the amount of proflavine present as cation (which depends on the pH of the medium and the pK_a of the drug) which controlled the bacterio-static effect (21, Fig. 4). While, in the experimental work reported here, no measurement was made of final pH of the medium following growth of the fungus (principally because of the very small amounts of medium used in growth chambers), the medium was at a pH of 6 ± 0.2 before addition of the drug and the drug was strongly buffered at pH 7.2. It therefore seems improbable that drastic changes in the pH of the medium could occur.

Permeability to the inhibitor may also be an important factor in resistance of the cells. Davis (22) has said that one of the possibly phenotypic mechanisms responsible for drug resistance is the decreased permeability of the cell (or of subcellular units) to the inhibitor. He says that permeation systems, like enzymes, can be gained or lost by mutation, and that it is easy to imagine that mutations, as well as physiological adaptations, could alter the number of units for transporting a drug, and hence could establish various characteristic ratios of internal to external free drug. Recent evidence has been produced (Pardee, Jacob and

Monod; 23) to demonstrate that the enzyme β - galactoside permease, one of the enzymes forming part of the permeation systems in certain micro-organisms, is genetically controlled. It is probable that other enzymes responsible for permeability to substrates are also controlled by genes.

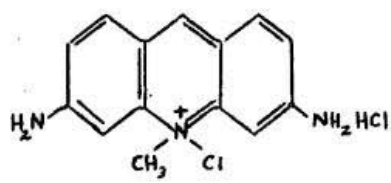
The theory that the drug could affect permeability is weakened by the finding of Jackson and Hinshelwood (24) that the time required for proflavine to exert its inhibitory action on Bact. lactis aerogenes was independent of the concentration gradient between the inside and outside of the cell.

As an explanation for proflavine-resistance in Bact.lactis aerogenes, Peacocke and Hinshelwood (25) submitted that there is a limit to the amount of this drug that the cells could take up, and thus immunity could be given to much higher concentrations than those on which the strain had been trained, since the cells would be impermeable to a portion of higher concentrations. It has been pointed out that, in the process of growth on medium containing proflavine, the hyphae of A. nidulans take on the colour of proflavine, indicating that the inhibitor is being absorbed by the mycelium. This effect was observed in trained and untrained strains.

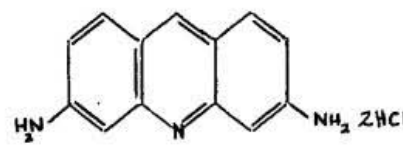
Two authors have discussed the problem of resistance to acridines in fungi and have produced evidence bearing on the experiments reported here. Roper and Kafer (6) deal with Acriflavine-resistant mutants of Aspergillus nidulans;

Bartlett (5) deals with the gain and loss of resistance in the fungus Penicillium roqueforti Thom. One of the drugs investigated by Bartlett was proflavine.

Roper and Kafer studied three mutant strains of A. nidulans, resistant in different degrees to acriflavine, a mixture of the hydrochloride of 2,8-diamino-10-methylacridinium chloride and 2,8-diaminoacridine. The formulae are taken from the Merck Index and are:



2,8-diamino-10-methylacridinium chloride



2,8-diaminoacridine

The mutants were selected by plating conidia of sensitive strains on the complete medium described by Pontcorvo (8) containing 0.005% (w/v) (50 ppm) acriflavine. This concentration was reported to be approximately three times that required to prevent germination of sensitive conidia plated at a density of 10^7 conidia per dish. They found that at such high densities some diffuse growth was obtained round the edges of the dishes were masses of conidia had clumped. They showed that this growth was due to adaptation and not mutation by transferring the conidia from such growth to further acriflavine medium on which it invariably failed to

grow. The vigorous growth of the mutant types distinguished them from growth due to adaptation. The size of the inoculum for this further test is not referred to.

The first mutant of Roper and Kafer was obtained from the plating of the strain paba 1 y; the second from ad 15 paba 1 y and the third from ad 1 pyro 5 sd bi 1. Several resistant colonies were obtained at each plating. No attempt was made by them to estimate accurately the frequency of acriflavine-resistant mutants, but the yield of resistant colonies in each selection varied between 1 in 2×10^7 and 1 in 10^8.

In the course of the experimental work reported here, an attempt was made to repeat the production of acriflavine-resistant mutants as reported by Roper and Kafer. It was found that a heavy inoculum (2×10^8) of sensitive spores of the mutant paba 1 y pyro 4 when plated on complete medium plus acriflavine (BDH) produced profuse but not confluent growth on 20 to 30 ppm; 8 colonies on 40 ppm; one colony on 50 ppm and one on 60 ppm. Growth of the organism did not appear on higher concentrations of the inhibitor. On reinoculating a small number of spores from the colony on 50 ppm to medium containing 50 ppm acriflavine, only one colony was produced. In a similar experiment in which approximately 2×10^2 spores of the sensitive mutant were

inoculated to the same range of concentrations of acriflavine, no growth was produced on concentrations greater than 20 ppm. On 20 ppm, 4 colonies were produced. On reinoculation of spores from one of these colonies to 20 and 30 ppm, no growth occurred.

The purpose of the experiments described above was to demonstrate the greater inhibitory effect of acriflavine at low concentrations (up to 60 ppm) than that exerted by proflavine at relatively higher concentrations (up to 400 ppm). It is obvious that those few colonies appearing on the higher concentrations of the inhibitor were not mutants, since their resistance was so shortlived,nor were they fully adapted to the presence of the inhibitor. Since it was not intended at this stage that a genetic analysis of the resistant mutant be attempted, no further attempts were made to produce mutants such as those described by Roper and Kafer.

In all experiments reported here, an inoculum of 10^2 conidia/dish was used, when a proflavine sensitive (untrained) was investigated to determine its native resistance to proflavine. It was found that the strain always grew on proflavine up to a concentration of 350 ppm. When growth on 375 ppm was obtained, it occurred after 91 hours incubation, and that on 400 ppm, after 112 hours. Sporulation occurred on all concentrations approximately 20 hours after appearance of colonies. Thus

inoculum containing only 10^2 conidia of the sensitive strain produced colonies which sporulated on proflavine concentrations up to 400 ppm. The difference between these results and the findings of Roper and Kafer upon inoculation of the sensitive strains is apparently more directly related to the fact that acriflavine has a greater inhibitory effect than does proflavine, than to the size of inoculum employed.

The size of inoculum can influence the apparent resistance of the spores to proflavine and, as will later be pointed out, to phenylmercuric acetate. A colleague, in a series of experiments (unpublished) conducted in this laboratory, concerning the training to drug resistance of the same strain used herein, found that with an inoculum containing an estimated 10^6 conidia, the sensitive strain was inherently resistant to concentrations of proflavine as high as 500 ppm.

The usual failure of the sensitive strain studied here to grow on petri plates with concentrations of proflavine higher than 350 ppm and its ability to germinate on 2000 ppm proflavine in growth chambers, may be due to the fact that the plates were inoculated with 10^2 conidia (on an area of approximately 65 $cm.^2$), the growth chambers with approximately 5×10^4 conidia (on an area of approximately 6.25 $cm.^2$).

A deliberate attempt was made, in the experimental work described here, to rule out the appearance of spontaneous mutants resistant to the inhibitor, such as those obtained by

Roper and Kafer, and to concentrate on a gradual adaptation to the presence of the inhibitor. Thus, a small inoculum was employed in transfers to Petri plates, and the germination of virtually all conidia involved were observed continuously in growth chamber experiments. At no point in the experiments recounted was there a rapid increase in resistance involving single conidia or even small numbers of conidia. It was felt, therefore, that any increase in resistance observed would be the result of adaptation, rather than spontaneous mutation.

Bartlett (5) found that resistance in Penicillium roqueforti Thom was rapidly acquired above 100 ppm proflavine, until it reached 430 ppm, where the drug was toxic to the mold. This is close to the level of resistance (475 ppm) retained by the proflavine-trained strain studied here.

He also noted that at concentrations of the drug above 100 ppm, the mycelium of the organism appeared to absorb a certain amount of the drug, and the hyphae appeared yellow. A similar occurrence has been noted in the sensitive and resistant strains studied here.

He goes on to say that microscopic examination showed that germination of inoculated spores was normal on most concentrations of the drug, but that growth of mycelium after germination was retarded or stopped entirely by very high

concentrations. In the experiments reported here, microscopic examination showed that the hyphae were initially normal and growth continued rapidly. This growth, on higher concentrations of the inhibitor, was retarded on extended incubation. As well, the colonies grown on Petri plates containing high concentrations of the drug were very confined, and on concentrations of 350 ppm and higher, hyphae were not prominent.

Bartlett further points out that in all cases of training and detraining of P. roqueforti Thom, confluent growth occurred, and that a very high proportion of the spores became simultaneously resistant to the inhibitor. He suggested that this would indicate an impossibly high mutation rate.

Roper and Kafer suggest that resistance is the result of a further mutation, at a definite position, of a single gene. They report, from crosses involving different resistant strains, that two of the mutant alleles are semi-dominant. Such an explanation is undoubtedly a valid explanation of their findings, since resistance occurred as relatively rare events.

Proflavine, as well as other acridines, has been described as a mutagen, and said to exert a chemical effect upon the genetic mechanism of cells. Roper and Kafer (6) showed that addition of nucleotides inhibited the action of

acriflavine. Oster (26) showed by his studies using fluorescence quenching as a guide, that the fluorescence of acriflavine is quenched by small amounts of nucleic acid. This quenching was due to complex formation between acriflavine and nucleic acid, altering the reduction potential of acriflavine. Hydrogen ions could replace the bound dye molecules. Schnitzer (27), in studying the resistance to drugs in Protozoa, found that antagonism of acriflavine to yeast nucleic acid could only be shown in vitro, but that proflavine was detoxified by nucleic acid. If the proflavine is attacking nucleotides, it is quite possible that it can affect the structure of DNA and RNA.

Sinsheimer (28) considered the effects of proflavine and various other chemical mutagens on the phage genome. The effects may be the results of the action of these mutagens in causing mistakes to be made during the replication of the DNA, thereby resulting in changes in the nucleotide sequence. Proflavine was found by him to increase the mutation rate at the rII locus some thirty-fold, and he says that proflavine mutations may represent replacement of a purine-pyrimidine pair by a pyrimidine-purine pair.

Caldwell and Hinshelwood (29), experimenting with Bact. lactis aerogenes, estimated the content per cell of RNA and DNA. Filaments and cells of twice the normal size were

produced, when unadapted cells underwent their first subculture at a proflavine concentration just below that required for bacteriostasis. If, for estimation of DNA per cell, a filament was counted as a single cell, the DNA content was found to be normal when compared with cells grown in several other drugs, or in the absence of proflavine and these other drugs. For estimation of RNA content per cell, if each filament was considered a single cell, the RNA content varied markedly from drug to drug; for proflavine it was very high, compared with the others. However, if each filament was taken to represent the equivalent larger number of single cells, then both the RNA and DNA content was lower than normal. The authors felt that the proflavine must, in some way, interfere with the duplication of the DNA structure.

Since proflavine, in company with other acridines, exerts a chemical effect upon nucleic acids, and can also be shown to act as a mutagen, it can be argued that in the process of action as a mutagen, proflavine may induce formation of mutants resistant to the action of the mutagen itself. Other evidence, however, indicates that proflavine may affect other chemical entities of the cell, as well as nucleic acids. Proflavine affects the dehydrogenase activity of Bact. lactis aerogenes (13), the cytochrome system of yeast and reduces catalase activity in Bact. lactis aerogenes (14). Since DPN (Co I) is a necessary part of all these systems (30), it is

possible that it is the production of DPN which is being thwarted.

On the basis that enzymes are attacked, it is possible that the elimination of nucleotides by proflavine means that this drug is attacking those enzymes which contain nucleotides. If these enzymes were protected by an excess of nucleotides (which combine with the drug), then the normal metabolism of the cell could continue. The number of spores present would help in this process, since they could absorb drug from the medium surrounding the chosen spore, and thus give it added protection by cutting down the concentration of the drug . The fact that proflavine can combine with nucleotides could explain the lowering of DNA and RNA content in the cells examined by Caldwell and Hinshelwood (29). The nucleotides necessary for the duplication of these would be attacked by the proflavine.

Phenylmercuric acetate was found to be much more toxic to A. nidulans than was proflavine. Although Russell (7) has stated that this inhibitor is toxic to most fungi in concentrations of 2-10 ppm phenylmercuric acetate, in the case of A. nidulans, a concentration of 0.20 ppm inhibits entirely the growth of the untrained mutant. The second strain employed was one which had been trained, at one time, to grow in the

presence of nearly six times this concentration. The trained strain used in the majority of experimental work described here, however, was trained to grow on concentrations of 0.50 ppm, and this concentration of phenylmercuric acetate was selected as the level at which most tests were made.

In certain respects the observed action of phenylmercuric acetate on the conidia of A. nidulans resembles that of proflavine. In the presence of both inhibitors, a reduced number of conidia of all strains (both trained and untrained) succeeded in completing the germination process. In both cases, too, germination was more successful among conidia in groups than in isolated conidia. This effect was much more pronounced in the presence of phenylmercuric acetate than in the presence of proflavine. However, whereas those conidia which did not germinate in the presence of proflavine retained the size and shape common to normal ungerminating spores, those conidia which did not succeed in germinating in the presence of phenylmercuric acetate frequently demonstrated abnormal shapes and sizes. Toxic concentration of phenylmercuric acetate inhibited entirely the swelling of conidia.

Once conidia had successfully germinated in the presence of either drug, it was observed, that hyphae were reduced in length, and the mass of mycelium present, both on petri plates and in growth chambers, was much less than that produced by the organism on drug-free medium.

In addition to these effects, phenylmercuric acetate caused a number of other observed phenomena not produced by proflavine. Principal among these was the presence of deposits in conidia which had successfully germinated, and in the hyphae produced by them. It was also noted that sporulation rarely occurred in untrained strains in the presence of 0.50 ppm phenylmercuric acetate and higher concentrations of the inhibitor, although sporulation was effected in trained strains of the organism. When colonies of the trained strain sporulated, it was observed that conidiophores were markedly reduced in length, although conidial heads appeared normal in size and arrangement. Whereas conidiophores on drug-free medium normally reach a length of approximately 100 μ (findings of Pontecorve; 8), the resistant strains studied here produced conidiophores of an average length of 50.5 μ in the presence of the inhibitor.

Thus, it would appear that phenylmercuric acetate is involved in all of the processes of the asexual life cycle of the organism. This would indicate that phenylmercuric acetate must exert a broad biochemical action upon the organism.

There are indications from literature, that mercuric compounds attack enzymes. Cook, Kreke, McDevitt and Bartlett (31) studied the effects of basic phenylmercuric nitrate on cytochrome oxidase, with ascorbic acid as the substrate. It

was shown that the compound was acting on the enzyme system and not directly on the substrate. The mercury compound in concentrations from 2.3×10^{-5} M. to 1.25×10^{-5} M. gave complete inhibition of the succinoxidase system. The compound also actively inhibited succinic dehydrogenase, although higher concentrations were required than with the oxidation enzyme preparation. It also inhibited lactic dehydrogenase. Glucose dehydrogenase activity was depressed, but not as readily as succinic or lactic dehydrogenase. It depressed the action of crystalline beef liver catalase.

Phenylmercuric nitrate was therefore considered to be a non-specific depressant of the enzyme systems studied. The authors note that mercury compounds are general protein precipitants, and they felt that the most obvious point of attack of the enzymes was the sulfhydryl group. This group is essential for succinic dehydrogenase activity. It had been reported that -SH groups were not essential for functioning of cytochrome oxidase and catalase.

Cook, Perisutti, and Walsh (32) studied the problem of whether the depression of yeast respiration by phenylmercuric nitrate could be prevented by sulfhydryl-containing compounds. They used a pure culture of Saccharomyces cerevisiae.

When 10^{-4} and 6×10^{-5} M. cysteine were present with

1.5×10^{-5} M. phenylmercuric nitrate, the depressing effects of the mercurial compound were lessened. dl-Homocysteine behaved similarly to cysteine. l-Cystine and dl-Methionine exerted no protective effect against the phenylmercuric nitrate.

The authors therefore concluded that depression of enzyme activity by basic phenylmercuric nitrate might involve interaction with the sulfhydryl groups of the enzymes.

Cook and Perisutti (33) showed that the basic phenylmercuric nitrate depression of cytochrome oxidase, like that of yeast respiration, could be prevented by the prior addition of sulfhydryl-containing compounds, but the depression of neither cytochrome oxidase nor yeast respiration could be reversed by subsequent addition of sulfhydryl-containing compounds. The authors concluded that the mercurial compound might react with -SH groups in enzymes, and that it might not be specific for such groups, but might also react with other groups present in the enzyme protein.

Thomas and Cook (34) found that the inhibition of the growth of Escherichia coli, Eberthella typhosa, and Staphylococcus aureus by basic phenylmercuric nitrate could be antagonized (diminished or prevented) by the addition of sulfhydryl-containing compounds (cysteine, glutathione, and homocysteine, but not cystine and methionine).

Kreke, Kroger and Cook (35) found that the succinoxidase

system was more sensitive than the anaerobic succinic dehydrogenase system to the inhibitory action of basic phenylmercuric nitrate, phenylmercuric hydroxide, and p-chloromercuribenzoic acid. They attributed this not only to a combination of the mercurial inhibitor with the sulfhydryl group of the dehydrogenase, but also to an effect on the protein particles of the enzyme preparation affecting the accessibility of the succinic dehydrogenase to cytochrome oxidase.

Seibert, Kreke and Cook (36) showed that partially purified cytochrome oxidase is also inhibited by the mercurials. The mechanism of action of mercurials was studies by spectral analysis. The type of inactivation was due to denaturation of the protein moiety of the enzyme.

Thus, mercurial compounds may be considered as non-specific depressants of enzyme activity, due to their ability to denature the protein moiety of the enzyme. They may be specific depressants, attacking the sulfhydryl groups present in the enzyme. The fact that they can depress the activities of enzymes is firmly established. Because of their non-specificity, they could attack many essential enzymes at the same time, thus killing the spores quickly. Since so many enzymes may be attacked, low concentrations of the drug would be all that is required to cause death. This fact is borne out by the findings herein. As low a concentration as 0.20 ppm phenylmercuric acetate was effective in preventing growth on petri plates of a small inoculum of sensitive spores.

That the trained strain has acquired increased resistance to the inhibitor is demonstrated in a number of ways: (1) ability to produce colonies on medium containing concentrations of phenylmercuric acetate normally toxic to the untrained strain; (2) the ability of conidia of the trained strain, both singly and in groups, to swell and produce germ tubes, on concentrations of phenylmercuric acetate which inhibited these processes in less-trained strains; and (3) the ability to sporulate on high concentrations, and thus to complete the asexual cycle. This ability is normally denied untrained strains by the action of the inhibitor.

As for the development of resistance to any other inhibitor, a number of explanations can be offered. Principal among these is a mutation, or a series of mutations, giving to the organism gradually increased resistance. There is some support for such a theory in the evidence presented earlier. Resistance was acquired gradually, and increased in a stepwise fashion, suggestive of the pattern of resistance to chloramphenicol and other drugs acquired by E. coli, which has been described as the effect upon polygenes by Cavalli and Maccacaro (2) and Bartlett and Hinshelwood (4). A relatively small proportion of the conidia were at first more resistant, and this resistance was transmitted to a larger proportion in succeeding generations. Other of

the observed facts, however, tend to indicate that mutation need not be the only available explanation.

A most interesting observation involved the appearance of deposits in the hyphae of conidia from both trained and untrained strains. When conidia germinated in the presence of phenylmercuric acetate, it has been noted that deposits occurred earlier in the hyphae of conidia from the trained strains than in those from the untrained strains. It is suggested that the appearance of these deposits can be closely linked with the ability to grow in the presence of phenylmercuric acetate. By some mechanism, phenylmercuric acetate may be deactivated, and may be absorbed by the mycelium in a form harmless to further growth of the organism. Resistance may then involve an increased ability to deactivate the mercurial.

A possible mechanism is suggested by Smalt, Kreke and Cook (37) who investigated the effects of a number of phenylmercuric compounds on certain enzyme systems. They found that all but one inhibited these enzymes to an extent quantitatively similar to phenylmercuric hydroxide. This suggested to them that the most effective mercurial compounds dissociate readily and act on the enzymes as the phenylmercuric ion. Resistance may therefore be the result of a chemical combination, which binds the phenylmercuric ion. Such a mechanism may possibly be directed by gene action.

Russell (7) has stated that although phenylmercuric acetate is highly toxic to many fungi at 2-10 ppm mercury, there is a strain of Penicillium roqueforti Thom which can tolerate very much higher concentrations. He showed experimentally that P. roqueforti isolated from pulp impregnated with phenylmercuric acetate could absorb mercury into its mycelium. After the mold had been grown for a month on liquid media containing 20 ppm of phenylmercuric acetate, the mycelium was filtered off, washed and analysed for mercury. About 75% of the weight of mercury found on analysis was present in the fungal pellicle. Such an absorption of the mercurial by P. roqueforti Thom would tend to strengthen the hypothesis that the hyphae of A. nidulans can absorb the phenylmercuric ion and possibly render it less toxic.

A thorough explanation for the observations presented here remains to be found. A principal tool for such studies might well be the techniques of genetic analysis devised by Pontecorvo, and making use of resistant strains gradually trained, rather than selected as mutants. A second approach, specific to the development of resistance to phenylmercuric acetate, might involve the use of radioactive mercury, and an attempt to determine by its use (and the micro-radioautographic techniques) if the 'deposits' are, in fact, a form of mercurial substance.

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