DRUG RESISTANCE AND MORPHOLOGICAL VARIATION IN PHYTOPHTHORA CACTORUM (LEB. & COHN.) SCHROET

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CONTENTS

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GENERAL INTRODUCTION

In recent years rapid progress has been made in the study of genetics by exploiting the unique characteristics of micro-organisms. Many fungi, like bacteria and viruses, are easily cultured, reproduce rapidly and abundantly but they also resemble higher plants and animals in having a well defined sexual cycle resulting in regular haploid and diploid phases. These features and the occurrence of heterokaryosis make the fungi admirable tools for the investigation of certain "fundamental" problems such as the nature of gene action, mechanisms of genetic exchange and cytoplasmic inheritance.

The Ascomycetes and to a lesser extent the Basidiomycetes have figured prominently in recent genetical studies and along with this has come a deeper understanding of their life history and general biology. By contrast the heterogeneous assemblage of fungi known as the Phycomycetes, has been neglected. Genetical analysis in this group has rarely been attempted possibly due to the fact that these fungi offered no special advantages for basic studies over the better known fungi with which rapid progress was being made. In fact certain characteristics, such as the frequent dormancy of the zygote and the absence of tetrads, would make them less desirable test organisms. As a result of this neglect, little is known of the nuclear phases

in the life-cycles or indeed of the nutrition, physiology or biochemistry of the Phycomycetes.

Nevertheless, a few reports have been made of genetical studies in the classes Zygomycetes and Chytridiomycetes. The remarkable early studies of Blakeslee (1906) on the inheritance of mating types were followed by the researches of Burgeff (1914) and Köhler (1935). Multinucleate gametangia fuse prior to zygospore formation but it is not yet clear what happens to the nuclei during maturation and germination of the zygospore. According to the cytological evidence obtained by Cutter (1942) nuclear behaviour varies from species to species, fusion and meiosis occurring in the zygospore or in the germ sporangium. The numbers of nuclei which fuse and undergo meiosis is also variable. Sjowall (1945) reported that some of the nuclei present in the zygospore may degenerate during the resting period. Further studies will be required to determine how many diploid zygotic nuclei undergo meiosis and whether all four products of each division survive and can be recovered. Recently heterokaryons between two auxotrophic mutants of the asexual species Rhizopus javanicus have been synthesised (Minami & Ikeda (1962).

An intensive study of members of the Blastocladiales, an order of the uniflagellate Chytridiomycetes, by Emerson & Wilson (1954), Cantino (1964) and others has given us a great deal of information about the life-cycles, cytology, physiology, biochemistry and morphogenesis of these water moulds. Cytogenetic evidence suggests that certain

species of Allomyces have alternating haploid and diploid phases in their life-cycle. Polyploid series exist within species such as Allomyces arbuscula where n = 8, 16, 24 or 32 (Emerson & Wilson 1954). Segregation of biochemical markers in Allomyces arbuscula (Yaw & Cutter 1951) indicates that gametophytes are haploid and that meiosis occurs in the resistant sporangia.

Although the Comycetes are of greater economic significance than either of the classes already mentioned our knowledge of the biology of this group is limited. Species of Pythium, Plasmopara and Phytophthora belonging to the Peronosporales are notable plant pathogens which cause serious damage to crop plants. It may seem rather surprising that we still do not know whether the somatic nuclei of these fungi are haploid, diploid or polyploid; mechanisms controlling variation are quite unknown. Genetical investigations have been thwarted by the persistent dormancy of the zygotic oospore which has prevented the sampling of large numbers of sexual progeny. However, Couch (1926) working with Dictyuchus was able to germinate oospores and obtain the progeny from matings between different strains. The segregation patterns found, indicate the presence of a complex mechanism controlling mating reaction which cannot be understood without further investigations. Many reports (Leonian 1926; Reddick & Wills 1938; Stamps 1953; Buddenhagen 1958; Thurston Wilde & Sudia 1959 and others) deal with observations made on variations in growth rate, morphology or pathogenicity in the genus Phytophthora but do not elucidate the mechanisms whereby such changes occur.

Recent cytological studies (Sansome 1961 and 1963) on Pythium debaryanum and Phytophthora erythroseptica would seem to indicate that meiotic division takes place in the gametangia prior to fertilization If this is indeed the case then the life-cycle in these species and possibly in all Oomycetes would be based on the diplontic pattern. As interpretation of division figures in these fungi is extremely difficult, genetical proof of this hypothesis is required.

A variety of sexual systems are to be found in the Oomycetes. Many species are homothallic, some are heterothallic while others exhibit a type of mating reaction known as relative sexuality. (Raper 1960; Couch 1926; Leonian 1931 and others). Little or nothing is known about the factors controlling sexuality or their inheritance.

It was decided that an attempt should be made to solve some of these problems posed by the oomycete fungi. The primary aim of this research project was to undertake a genetic analysis with a suitable fungus to determine the ploidy of its somatic nuclei and, as far as possible, the mechanisms controlling variation.

Phytophthora cactorum was chosen as the test organism for a number of reasons:

(1) A member of the Peronosporales was chosen because of the economic significance of this group of phytopathogens.

(2) The genus Phytophthora includes around seventy species; all are non-obligate parasites of higher plants and many are responsible for serious damage to cultivated plants involving many countries in large economic losses. e.g. P. infestans, P. parasitica, P. palmivora, P. fragariae. In the past few decades our knowledge of genetically determined resistance and its inheritance in host plants has increased steadily (Miller 1953; Black & Gallegly 1957 and others) but the origin and nature of race changes enabling the pathogen to attack new resistant hybrid hosts still remains obscure (Mills & Peterson 1952; Gallegly & Nichenmuller 1959 and others).

(3) A homothallic species was chosen because it is still doubtful if the oospores formed in paired cultures of the so-called heterothallic species do in fact result from a fusion between a gametangium from each parent. In addition, oospores produced in paired cultures have never been germinated. Pontecorvo (1953) has shown it is quite possible to carry out a genetical analysis using a homothallic fungus.

(4) P. cactorum was chosen as its oospores have been germinated (Blackwell 1943b) and its life-history is known in some detail (Blackwell 1943a).

(5) Reports of spontaneous and induced morphological variation in this species indicate the need for further investigation (Stamps 1953; Buddenhagen 1958).

Before a meiotic analysis can be undertaken we must be able to control the growth and reproduction of the organism in pure culture

DRUG RESISTANCE AND MORPHOLOGICAL VARIATION IN *PHYTOPHTHORA CACTORUM* (LEB. AND COHN.) SCHROET.

SUMMARY

A genetical study has been made of the homothallic phycomycete fungus *Phytophthora cactorum*.

In the first section of the thesis techniques were developed for the production of asexual and sexual progeny from zoospores and oospores respectively. The survival of zoospores was found to be greatly improved if they were suspended in a solution containing 2% sucrose and Ringer's physiological salts rather than in water. The persistent dormancy of the oospores, which has thwarted genetical work with these fungi, was broken by passing them through the gut of the water snail *Planorbis* sp. Digested spores were surface sterilized in mercuric chloride, plated on non-nutrient agar and incubated four days in the light. By this means 66% germination of 25 day old oospores was obtained. In the absence of light germination was only 12%

Section II deals with the search for drug resistant mutants. Eighteen common antibiotics, antimetabolites and other toxic substances were screened for their ability to inhibit mycelial growth and zoospore germination.

Eight strains with increased tolerance to streptomycin were

isolated when zoospores of the wild-type were incubated in drugged liquid medium. Some were resistant to 1000µg/ml streptomycin, i.e. ten times that tolerated by wild-type. One strain had an absolute requirement for streptomycin. The drug tolerance of the asexual and sexual progeny (self-fertilised) of one resistant strain, Sr, and the dependent strain, Sd, was examined. The resistance or dependence was inherited in each case unchanged and there was no evidence of segregation of individuals with a differing response. Absence of segregation could be taken to indicate either that the somatic nuclei are haploid or that resistance and dependence are determined extrachromosomally. Evidence was obtained which indicated that the presence of large numbers of streptomycin sensitive zoospores in drugged media inhibited the growth of added zoospores of the Sr strains. Non-dependent but resistant growths arose in the Sd strain when it was cultured on drug-free medium.

Cultures with increased tolerance to sulphanilamide and acti-dione were obtained but resistance was found to be temporary and disappeared after growth in drug-free medium. Attempts to induce stable drug resistance with ultraviolet irradiation were not successful.

In Section III changes in colony morphology and their inheritance are described. Variation in colony morphology was found to occur spontaneously and could also be induced with a high frequency on irradiation of zoospores with ultraviolet light. Treatment of zoospores for short periods with streptomycin induced unstable changes in morpholo

reversion to wild-type always occurred in sub-cultures.

An interesting pattern of somatic segregation of morphological characters was found which appeared to be determined extrachromosomally although proof of this will be obtained only when the observed segregation is compared with the behaviour of known nuclear markers. This variation first occurred in the streptomycin dependent strain but continued on its reversion to streptomycin independence. Colonies with wild-type morphology, derived from single uni-nucleate zoospores, gave rise to asexual progeny containing a proportion of fast growing, diffuse segregants. Wild-type colonies reappeared in the asexual progeny of these segregants with low frequency. Single zoospores of the segregating line could be classified into two main groups according to the phenotype present in their asexual progeny. One interpretation suggested was that the zoospore contains a single determinant which exists in either of two forms and which mutates frequently from one form to the other. The proportion of the two phenotypes in the asexual progeny was found to change with ageing of the culture. The sexual progenies of these variants segregated in a similar way to the asexual progenies.

so that large numbers of asexual and sexual progeny can be produced. Easily characterised nuclear markers are also required. Section I of this thesis deals with the development of cultural techniques which allow reproduction to be controlled. Section II is devoted to methods used in the isolation of nuclear markers and to an analysis of the inheritance of the mutant phenotypes. Section III deals with the patterns of inheritance shown by certain morphological variants.

SECTION I

CULTURAL TECHNIQUES

Part I. The Growth of *Phytophthora cactorum* in pure culture.

The isolate on which the present study was made was obtained from the Commonwealth Mycological Institute, Kew (Serial number IMI.21168). The isolate has been cultured on oatmeal agar for many years and has been sub-cultured regularly. Abundant sporangia and oospores are produced by this isolate under suitable conditions.

In any study of variation, cultural conditions require to be kept as constant as possible. The morphology and growth rate of fungi vary with the physical and chemical composition of the environment. Variation induced by the environment must be reduced to a minimum by standardising the variable factors as much as possible. e.g. by cultivation in incubators to standardize temperature, by cultivation in glassware of a standard size and shape preventing variable loss of moisture and the use of media of uniform composition preventing variation due to nutritional differences.

Standardization of media.

Natural and synthetic media have been used in the present study. It is easy to obtain good vegetative growth by culture in certain synthetic media but the formation of reproductive structures in the desired numbers is most conveniently obtained on natural or semi-synthetic media.

Oat-meal agar. 100 gm. oat-meal
1,000 ml. de-ionised water
8.0 gm. 'Oxoid' No. 3 agar

The medium is autoclaved at 15 lbs/sq.in. for 15 minutes.

This is a useful natural medium for the maintenance of stock cultures. Variant strains are stored in one-ounce bottles containing 5 ml. of oat-meal agar. After incubation at 24°C for one week 10 ml. of sterile paraffin oil is added to each one-ounce bottle slope and cultures are then stored at room temperature. Where inocula of a particular strain are required regularly over a number of months, inocula are removed at monthly intervals from the original oiled cultures to minimise selection which would tend to take place more easily if inocula were taken from material kept actively growing by regular sub-culture. Oiled cultures stored at room temperature have provided uniform inocula of some strains for some two years. Growth on oat-meal agar is relatively slow and oospores and a few sporangia are formed. This medium has been used to provide oospores but it was found that they could be produced more easily in other media.

Pea-meal agar. 300 gm. Frozen Garden Peas (Blended)
1,000 ml. de-ionised water
8.0 gm. 'Oxoid' No. 3 agar

The medium is autoclaved at 15 lbs/sq.in. for 20 minutes.

This rich medium supports vigorous mycelial growth. Plates are covered with a dense mycelial mat after 8 days' incubation at 24°C. Growth is initially vegetative although oospores are produced

on the surface and embedded in the agar in old cultures. Pea-meal agar cultures provide a suitable source of actively growing mycelium for use in the production of the asexual spores (see Part II).

Synthetic medium.

In the present study it was not essential to grow the organism on a defined minimal medium as it would have been if auxotropic mutants were being produced. However it is essential to grow the organism on a standardised medium on which growth and morphological characteristics can be compared. Of the various media tested one containing sucrose, asparagine and thiamin was found to support the best growth. The medium used was the same as that used by Elliott et al (1964)

Composition of the minimal medium.

Sucrose		10.0 gm.
L-asparagine		1.0 gm.
magnesium sulphate	$MgSO_4.7H_2O$	0.25 gm.
potassium dihydrogen phosphate	KH_2PO_4	0.5 gm.
thiamin hydrochloride		1.0 mg.
trace element solution		1 ml.
de-ionised water		1,000 ml.

Composition of trace element solution.

$Na_2B_4O_7.10H_2O$	88 mg.	$Na_2MoO_4.2H_2O$	50 mg.
$CuSO_4.5H_2O$	393 mg.	$ZnSO_4.7H_2O$	4,430 mg.
$Fe_2(SO_4)_3.5H_2O$	910 mg.	EDTA	5 gm.
$MnCl_2.4H_2O$	72 mg.	Water	1,000 ml.

The medium was autoclaved at 15 lbs/sq.in. for 15 minutes and had a pH of 5.0.

Solid medium is prepared by adding 8.0 gm. of agar per litre of medium before autoclaving. It was found that growth rate and colony morphology varied considerably with the brand of agar being used. "Oxoid" agar No. 3 was chosen as the linear growth rate on minimal medium solidified with this agar was higher than that obtained using other agar brands or silica gel. The morphological differences between isolates appeared particularly distinct on this agar. The enhanced rate of growth could be due to the presence of vitamins or trace elements which have been shown to be present in agar (Leonian & Lilly 1940; Day 1942).

A semi-synthetic medium for germinating zoospores.

On plating zoospores suspended in 2% sucrose on plates of minimal medium it was found that the spores did not germinate (Part II of this section deals with the production of zoospores.). It was thought that zoospore germination might have additional nutritional requirements and so the germination of zoospores was compared on minimal medium and on two supplemented media. The media used were:

1) Minimal medium agar.
2) Minimal medium agar supplemented with an extract of wheat germ. 5 gm. of wheat germ ("Froment") was extracted by boiling five minutes in 200 ml. de-ionised water. 100 ml. of this extract was added to 100 ml. of twice concentrated minimal medium.
3) Minimal medium agar supplemented with an aqueous extract of garden peas. 150 gm. of frozen peas

were brought to the boil in 1,000 ml. of de-ionised water. The peas were filtered off and the clear liquid was added to an equal volume of twice concentrated minimal medium.

All three media were gelled with 8.0 gm/litre of "Oxoid" agar No. 3. Three plates of each medium were spread with 0.2 ml. of a zoospore suspension (10^4 spores/ml.) in 2% sucrose. Cultures were incubated for 6 hours when counts were made of the number of germinating spores. Spores with germ tubes over 40μ long were scored as germinated spores. One hundred spores were scored in each plate and the percentage germination recorded is shown in Table 1.

		Percentage germination per plate			
		Replicate 1	Replicate 2	Replicate 3	Mean
Medium	Minimal Medium	0	1	2	1
	M. M. + Wheat germ	3	2	4	3
	M. M. + Pea-water	51	50	54	51.7

Table 1. The percentage germination of zoospores plated on different media.

The results indicate that the aqueous extract of garden peas contains substances which stimulate zoospore germination.

Minimal medium supplemented with pea-water has been used as a standard medium throughout this study and will be subsequently referred to as standard medium agar or S.M.A. The pH of this supplemented minimal medium was 5.5. The active agent or agents in the pea extract are unknown and/so the nutrients present in the medium are not accurately

known but it can be prepared easily and its composition does not vary between different batches. Also, it is unnecessary if not undesirable to use a minimal medium in the isolation and characterisation of variants, some of which could be auxotrophic.

The standardised method for the preparation of S.M.A.

Minimal medium is prepared at four times the concentration given on page 9 and is dispensed into 1 litre or 500 ml. polythene bottles. Aqueous pea extract (300 gm. peas/litre) is also dispensed into polythene bottles. The bottles are stored at -45°C and when medium is required a bottle of each medium is thawed in hot water, the media are mixed and an equal volume of de-ionised water is added thus bringing the final concentration to the usual 10 gm/litre of sucrose and proportionate concentrations of the other constituents. The medium can then be dispensed into flasks, agar added if required (8.0 gm/litre "Oxoid" No. 3), and autoclaved at 15 lbs/sq.in. for 15 minutes. The freezing procedure avoids autoclaving of the stock solutions before storage. It has been shown that products toxic to Phytophthora spp. may accumulate by interaction of sugars and nitrogenous compounds during autoclaving especially when media are concentrated (Margolin 1942; McKaen 1956).

Part II. The Production of asexual progeny.

Introduction and Review of the Literature

In any genetical study it is of great advantage to be able to sample single nuclei from the growing organisms. This can be done by the use of uni-nucleate asexual spores. Fortunately, in Phytophthora spp. sporangia can be induced to release uni-nucleate zoospores which germinate immediately to produce daughter colonies. A review of asexual reproduction in water moulds by Waterhouse (1962) refers to most studies of any significance. A detailed description of morphological aspects of asexual reproduction in P. cactorum is given by Blackwell (1943a). Pyriform, papillate, multi-nucleate structures known as sporangia are formed terminally on vegetative hyphae under suitable conditions and usually remain attached to the parent mycelium. Sporangia may, under certain environmental conditions, initiate further vegetative growth by the formation of one or a number of germ tubes. This is often referred to as direct germination of the sporangium. Alternatively under different environmental conditions, cleavage of the protoplast of the sporangium takes place and the papilla breaks down to allow the release of zoospores, the uni-nucleate, biflagellate, naked swimmers. This pattern of zoospore formation is known as indirect germination of the sporangium. Such sporangia able to form zoospores are known as zoosporangia. The free-swimming zoospore eventually settles, encysts and immediately initiates a germ tube. Resistant sporangia and multi-nucleate chlamydospores may also be formed. Marks (1963) reports

that sporangia of P. infestans have an average of 6.2 nuclei which are thought to enter the sporangium from the sporangiophore before the cross-wall is laid down. Nuclear division was not observed in the sporangium.

The little we know of the physiology of sporulation and zoospore formation in Phytophthora has come from isolated studies carried out by different researchers working with different species. One of the most comprehensive accounts is that by Gooding and Lucas (1959). It emerges that certain factors are of outstanding importance in influencing the formation of sporangia.

1) The age of the vegetative mycelium. A young actively growing mycelium responds to induction better than an old and inactive one.

2) The nutrient status of the medium. Klebs (1899) showed that Saprolegnia spp. grow vegetatively on concentrated culture media but sporulate when mycelium is transferred to a dilute medium or to water. More recently, Schmitthenner (1959) working with Phytophthora spp. showed that growth is strictly vegetative on various concentrated natural media but that sporangia are formed in increasing quantity on increasingly dilute media. The effects of medium concentration have not been investigated further. The presence of inorganic ions in certain concentrations was shown to stimulate sporangium formation (Wills 1954; Gooding & Lucas 1959).

3) Water Relations. According to Schmitthenner (1959) the presence of free water is essential for sporangium formation in some species. Others including P. cactorum form sporangia on solid agar media.

4) Aeration. Leonian (1925) has said that "the chief limiting factor in the reproduction of Phytophthora is access to atmospheric oxygen". Gooding and Lucas (1959) found that the formation of sporangia in P. parasitica nicotianae is affected by the depth to which the mycelium is submerged in the liquid medium. They report optimal sporulation when the mycelium is kept wetted but is not submerged.

The contents of newly formed sporangia of Phytophthora spp. can be induced to cleave into zoospores by flooding cultures with water at a temperature below the optimum for vegetative growth.(Melhus 1911; Fawcett & Klotz 1934). Zoospores are released through a pore which arises in the apical papilla.

The Method used to produce zoosporangia and zoospores.

In the present investigation it is important to be able to produce large numbers of zoospores from sporangia of a reasonably uniform age. As already noted, growth is vegetative on S.M.A. but sporangia are formed on dilute S.M.A. However, the age of these sporangia is very variable and the numbers formed are small. Sporangia of a more uniform age are produced when strips of vegetative mycelium cut from

a colony on S.M.A. are transferred to soft non-nutrient agar (8.0 gm. "Oxoid" No. 3 agar, 1 litre de-ionised water), and incubated at 24°C for 72 hours. Sporangia are much more abundant when the mycelial strips are cut from a colony growing on pea-meal agar. The method adopted for the production of large numbers of zoosporangia of a uniform age is as follows:-

1) A pea-meal agar plate (30 ml medium per plate) is inoculated centrally with a 4 mm disc of actively growing mycelium and incubated for 8 to 10 days, by which time the colony has grown to the edge of the plate.

2) Three strips of the mycelial mat, approximately 0.5 cms x 6 cms, are cut from the surface of the pea-meal culture and are transferred to a plate of non-nutrient agar and the plate is incubated for 72 hours at 24°C.

To induce zoospores, a freshly incubated strip culture is flooded with de-ionised water at 15°C and is further incubated at 15°C. The first zoospores are released after 25 minutes and suspensions of between 10^4 and 5×10^5 zoospores/ml can be withdrawn after 45 minutes.

Counts of zoospores were made using a "Thoma" haemocytometer. One drop (approximately 0.05 ml) of suspension is placed on the graduated portion of the haemocytometer slide. The coverslip is lowered and pressed firmly onto the slide. It was found that large numbers of actively swimming zoospores were difficult to count but the zoospores can be immobilised by increasing the intensity of the light illuminating the slide for ten minutes. This causes encystment of the zoospores

which can then be easily counted. The number of spores per large square (1 sq.mm. in area and 0.1 mm. in depth) is counted. The average number ($\underline{x}$) per large square indicates a concentration of $\underline{x} \times 10^4$ spores/ml. Where an estimate of greater accuracy is required the numbers present in ten large squares, each prepared from a different drop of suspension, is determined.

The Survival of Zoospores in Suspension.

As it is necessary to be able to work with spore suspensions of known viability, tests were made to determine the percentage germination of zoospores produced by the above method when plated on S.M.A. The viability of zoospore samples was estimated by spreading 0.2 ml of dilutions of a counted suspension on plates of S.M.A. and counting the developing colonies after 72 hours. Disintegration of many of the ungerminated spores makes it undesirable to determine the viability by counting numbers of germinated and ungerminated spores. Estimates of the percentage germination in a number of separate platings were low and variable ranging from 0% to 30%. When zoospores suspended in de-ionised water were examined it was found that many were burst or disorganised and apparently dead.

Survival of zoospores in sucrose solutions.

Gooding and Lucas (1959) showed that viability of zoospores of Phytophthora parasitica nicotianae could be increased by adding sucrose to the suspending fluid. Zoospores suspended in one percent

	Replicates	Concentration of Sucrose : gm/100 ml					
		0	1	2	3	4	5
Numbers of	1	0	35	70	57	33	25
Colonies	2	0	42	55	62	42	38
per plate	3	0	43	60	48	39	20

Table 2. Numbers of colonies in platings of spores suspended in different concentrations of sucrose solution.

Source	Df	Mean Square
Between sucrose concentrations		
Linear	1	20.492
Quadratic	1	69.605
Cubic	1	10.809
Remainder	2	0.064
Within replicate plates	12	0.243

Table 3. Analysis of Variance: Colony establishment from zoospores suspended in different concentrations of sucrose.

aqueous sucrose solution were motile for longer periods and subsequently had a higher percentage germination than zoospores suspended in tap water or in Hoagland and Arnon's nutrient solution. It seemed likely that the observed bursting of the naked zoospore was due to the extremely low osmotic pressure of the de-ionised water used as suspending fluid and that the lysis could be prevented by increasing the osmotic pressure with sucrose. Accordingly, an experiment was designed to determine the viability of spores suspended in solutions of sucrose in de-ionised water of various concentrations. One ml. samples of zoospores in de-ionised water (6×10^4 zoospores/ml) were added to 9 ml. of 0%; 1%; 2%; 3%; 4% and 5% solutions of sucrose. The suspensions were further diluted 1 in 10 into the appropriate concentration of sucrose. Three 0.2 ml samples were removed from each of the diluted suspensions, plated on S.M.A. and incubated 72 hours when colony counts were made. The numbers of colonies, y, developing on plates with different concentrations of sucrose, x, are shown in Table 2. These data were transformed to $(y + \frac{1}{2})^{\frac{1}{2}}$ and an analysis of variance, Table 3, indicates that an equation of the third degree adequately represents the relationship between the quantities. The calculated regression line is shown in Fig. 1, and from the equation it was calculated that the optimal concentration of sucrose was 2.15 gm/100 ml. Since the viability in 2% sucrose does not differ significantly from the maximum viability expected in 2.15% sucrose, the former concentration was used in further investigations.

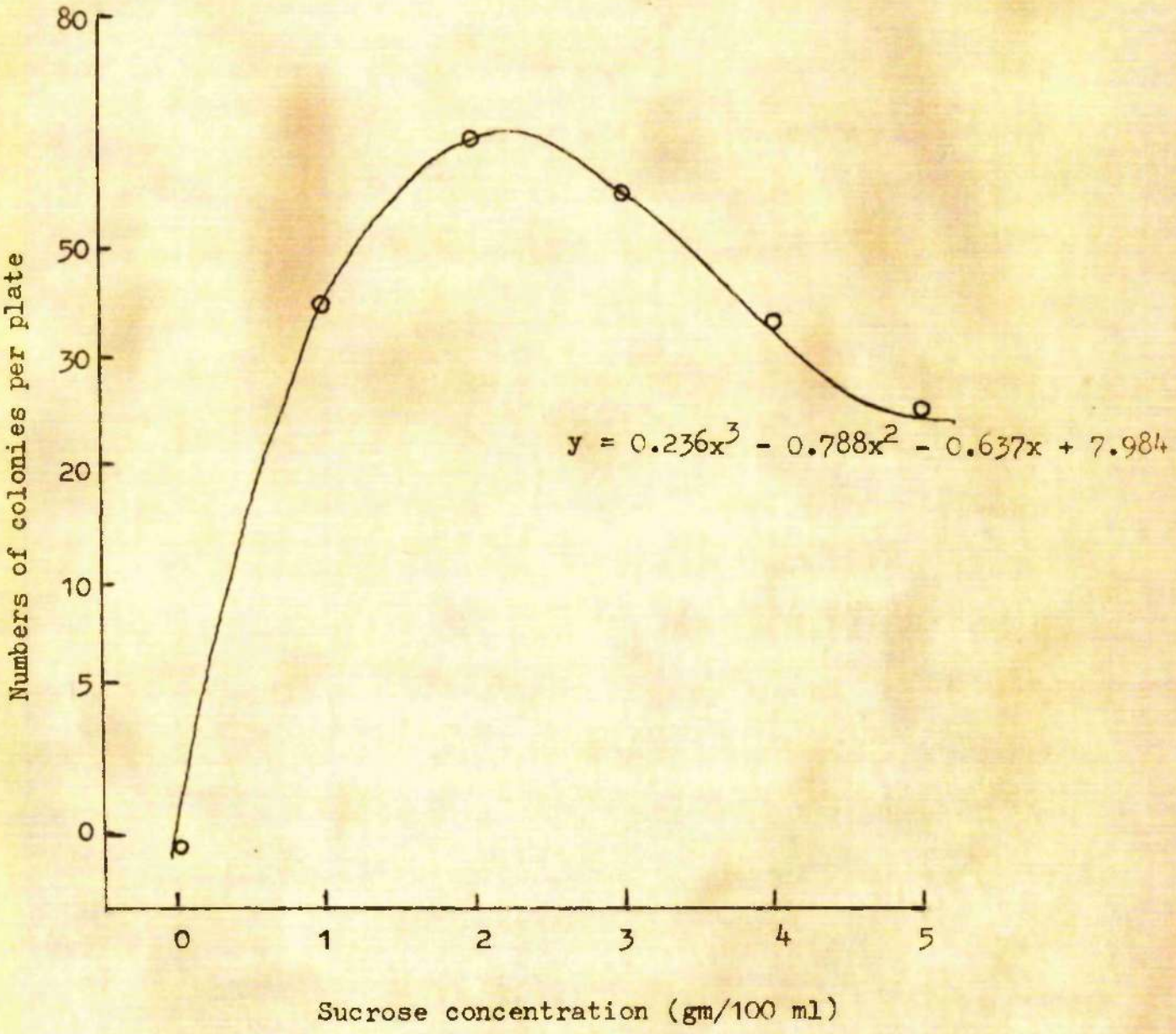


Fig. 1. Calculated curve representing the relationship between sucrose concentration and colony development.

Survival can thus be increased by adding suitable amounts of sucrose to spore suspensions but a proportion of spores was observed to be damaged before suspensions in de-ionised water were removed from strip cultures. When 2% sucrose is used to flood the cultures instead of de-ionised water, bursting is never observed but zoospore release is inhibited. Klebs (1899) reported a similar effect with Saprolegnia sp. Zoospore discharge was best, he said, in charcoal water and was suppressed by very low concentrations of nutrients or moderate concentrations (0.1% or less) of inorganic salts.

To ensure good zoospore release but at the same time prevent lysis, the method of making suspensions was therefore modified as follows: Cultures are flooded with 5 ml. de-ionised water at 15°C and 2.5 ml of 4% sucrose at 15°C is added to each after 30 minutes when the first zoospores are being discharged. Another 2.5 ml of 4% sucrose is added 45 minutes after the initial flooding. Suspensions now in 2% sucrose have concentrations ranging from 10^4 to 10^6 zoospores/ml. After the first suspension has been removed, the strip culture can be transferred to room temperature (18 - 22°C) and flooded with 10 ml of 2% sucrose. Suspensions as concentrated as the first can be obtained within 5 minutes. Such "second suspensions" will contain zoospores of a more uniform age, all having been released from sporangia within 5 minutes.

Later it was found that the viability of spores diluted into 2% sucrose from a concentrated suspension varied considerably and

that consistent counts of the percentage germination of zoospores seeded in plates at different densities could not be obtained. It was suspected that viability was being affected by the length of time for which spores were being held in suspension in 2% sucrose. To test this hypothesis the viability of spores suspended in 2% sucrose at different dilutions was compared over a short period of time. A "second suspension" of zoospores (3.5×10^4/ml) in 2% sucrose was immediately serially diluted by 1 in 10 three times into 2% sucrose, to give suspensions containing 3,500, 350 and 35 spores/ml. Three 0.2 ml samples were withdrawn from the concentrated suspension and from each dilution immediately and spread on plates of S.M.A. Six samples were taken from the 1/1000 dilution. Suspensions were stored at room temperature for 30 minutes when samples were again spread. The numbers of germinating spores per plate was determined as follows:

1) For plates seeded with samples of the most concentrated suspensions an estimate was made after 6 hours incubation by counting the spores germinating in twenty microscope fields (x 10 objective and x 10 eyepiece) and multiplying the total number by 141. (Area of 20 fields = $\frac{\text{Area of agar surface}}{141}$) The 20 fields were taken at random from all parts of the plate.

2) Total germination in plates seeded with the 1/10 dilution was determined after 12 hours incubation using a binocular dissecting microscope.

3) Colony counts were made in plates seeded from the 1/100 and 1/1000 dilutions after 72 hours incubation.

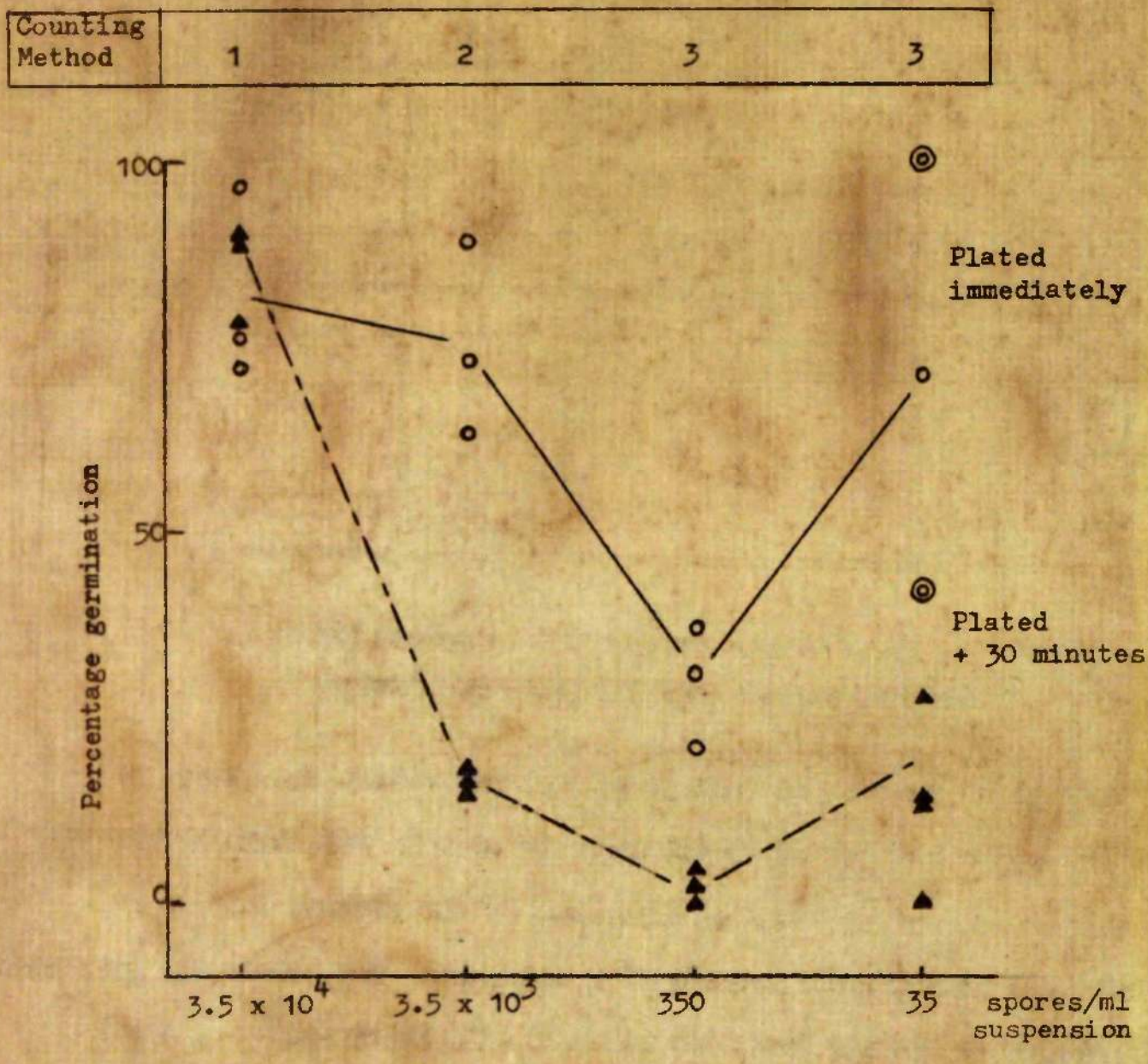


Fig. 2. The percentage germination of zoospores suspended in 2% Sucrose.

The number of spores germinating in each plate is recorded in Table 4.

		Spores/ml in suspension				
		3.5×10^4	3.5×10^3	350	35	
Plated	Replicates	6772	512	26	5	7
Zero		5070	623	22	7	3
Time		5362	445	18	3	5
Plated	Replicates	6349	116	3	2	0
+ 30		5503	117	2	1	0
mins.		6279	126	0	0	1

Table 4. Percentage germination of spores plated in 2% sucrose immediately and after 30 minutes.

The variation in percentage germination evident in dilutions of the suspension plated immediately and also in dilutions plated after 30 minutes is possibly influenced by the plating density or error due to different methods of estimations. The percentage germination at different plating densities is dealt with later (page 34. However it is obvious from Fig. 2 that the estimated percentage germination of spores stored in the diluted suspensions is significantly lower than that of spores plated immediately from the same suspensions, whether the estimate was made by method 2 or 3. The percentage germination

of spores from the undiluted suspension, estimated by method 1, does not fall with storage indicating that the effect is only apparent in more dilute suspensions. It is interesting that Gooding and Lucas (1959) found that zoospores of P. parasitica nicotianae were motile for the longest time in the most concentrated spore suspensions in distilled water or in 0.01 M sucrose solution.

Survival of zoospores in physiological salt solution.

It is not surprising that pure sucrose dissolved in de-ionised water does not provide a suitable environment for the survival of naked protoplasts. It is well known that many unprotected cells can only survive in a milieu with a favourable balance of inorganic ions. The complete survival in concentrated suspensions might conceivably be due to the presence of suitable ions which could have been derived from the strip culture, from the mycelium, from the sporangia or from the zoospores themselves.

It was decided to find out whether a physiological salt solution could be used to allow survival of zoospores for periods of a few hours. Ringer's salt solution, commonly used for bacterial suspensions was selected. It has the following composition: (Collins 1964)

NaCl	2.15 gm	$CaCl_2$	0.12 gm
KCl	0.075 gm	De-ionised water	1 litre
$Na_2B_2O_3 \cdot 5H_2O$	0.5 gm.	pH =	6.6

The survival of spores in dilutions made in Ringer's salt solution and in 2% sucrose was compared in the following experiment. A suspension

		Spores per ml in suspension				
		8.3×10^5	8.3×10^4	8.3×10^3	830	
		Estimate	Estimate	Estimate	Direct Count	
Diluted into 2% sucrose						
Zero Time	Replicates	146,943	15,662	392	115	72
		115,105	17,073	523	123	101
		178,781	16,908	504	75	100
+ 30 mins.		142,045	16,085	0	6	9
		129,800	15,521	1	7	8
		156,739	15,097	0	9	5
Diluted into Ringer's Salt Solution						
Zero Time	Replicates	—	10,018	1,277	96	81
		—	9,594	1,255	90	83
		—	13,263	1,248	96	71
+ 30 mins.		—	13,545	1,100	89	71
		—	10,300	1,191	66	109
		—	8,748	1,255	84	80

Table 5. Viability counts of spores diluted into 2% sucrose and into Ringer's solution, plated immediately and after 30 minutes.

of zoospores (8.2×10^5/ml) in 2% sucrose was serially diluted 1 in 10 into Ringer's solution and into 2% sucrose. 0.2 ml samples of each suspension were plated immediately and again after storage for 30 minutes at room temperature. Estimates and counts of germinated spores were made as detailed for the last experiment. The numbers of spores germinating in the various plates is recorded in Table 5. The percentage germination of spores in each plate was calculated and plotted against the suspension concentrations (Fig. 3). The results indicate that the viability of zoospores in dilute suspensions in Ringer's salt solution is not lowered as that of similar suspensions in 2% sucrose is, when stored at room temperature for 30 minutes. It should be noted however, that as dilutions were made from the concentrated suspensions in 2% sucrose, the dilution series in Ringer's Solution had decreasing amounts of sucrose present.

Values for the osmotic pressures of the suspending solutions and liquid standard medium were determined by the depression of the freezing-point method and are tabulated below. The similar values obtained for the three solutions would indicate that the poor survival in sucrose

Solution	Δf	O.P.(atmos)
2% Sucrose	0.122	1.47
Ringer's Solution	0.156	1.88
Liquid Standard Medium	0.119	1.52

Table 6. Osmotic pressures of suspending solutions and standard medium.

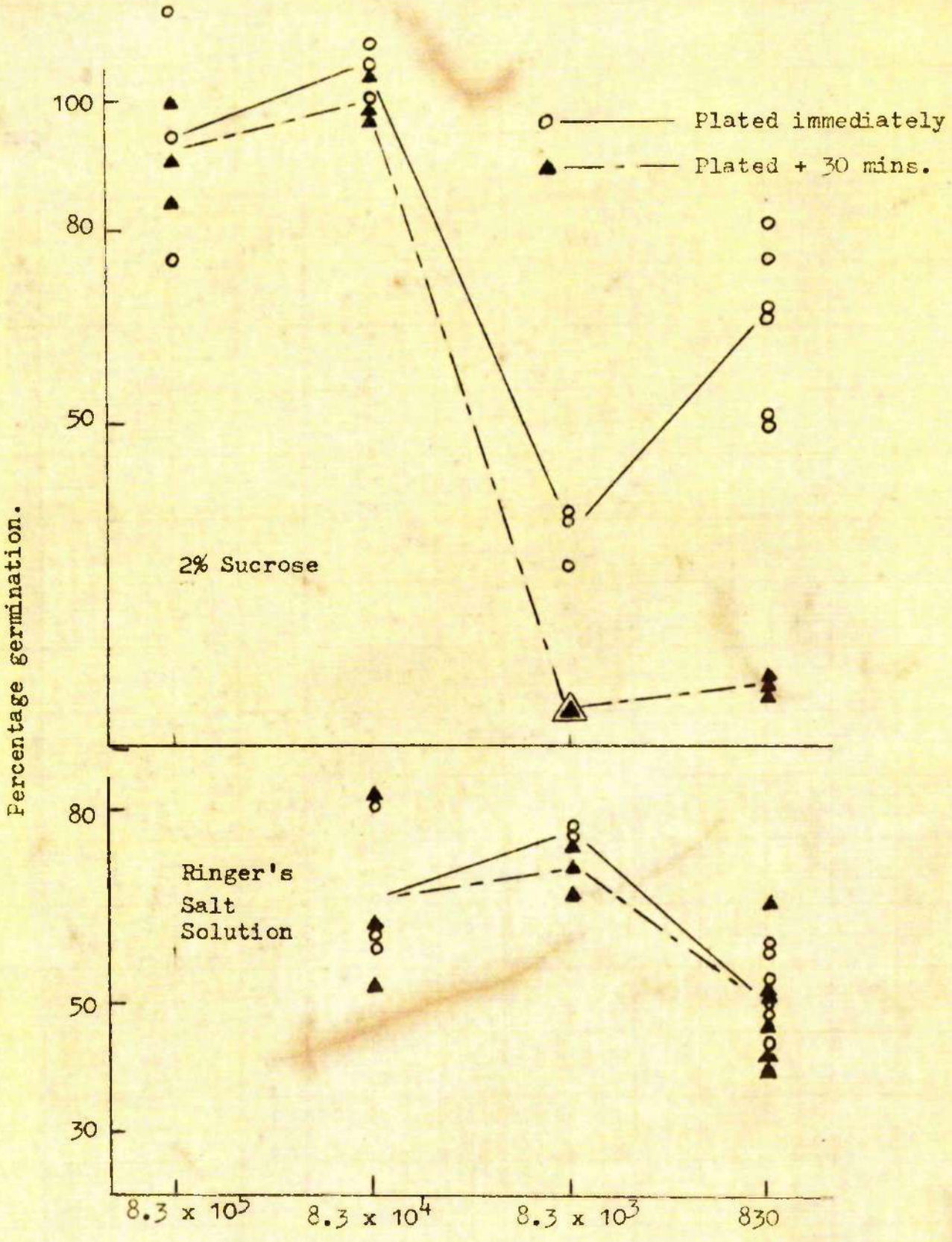


Fig. 3. A comparison of the survival of zoospores stored at different dilutions in 2% sucrose and in Ringer's Salt Solution.

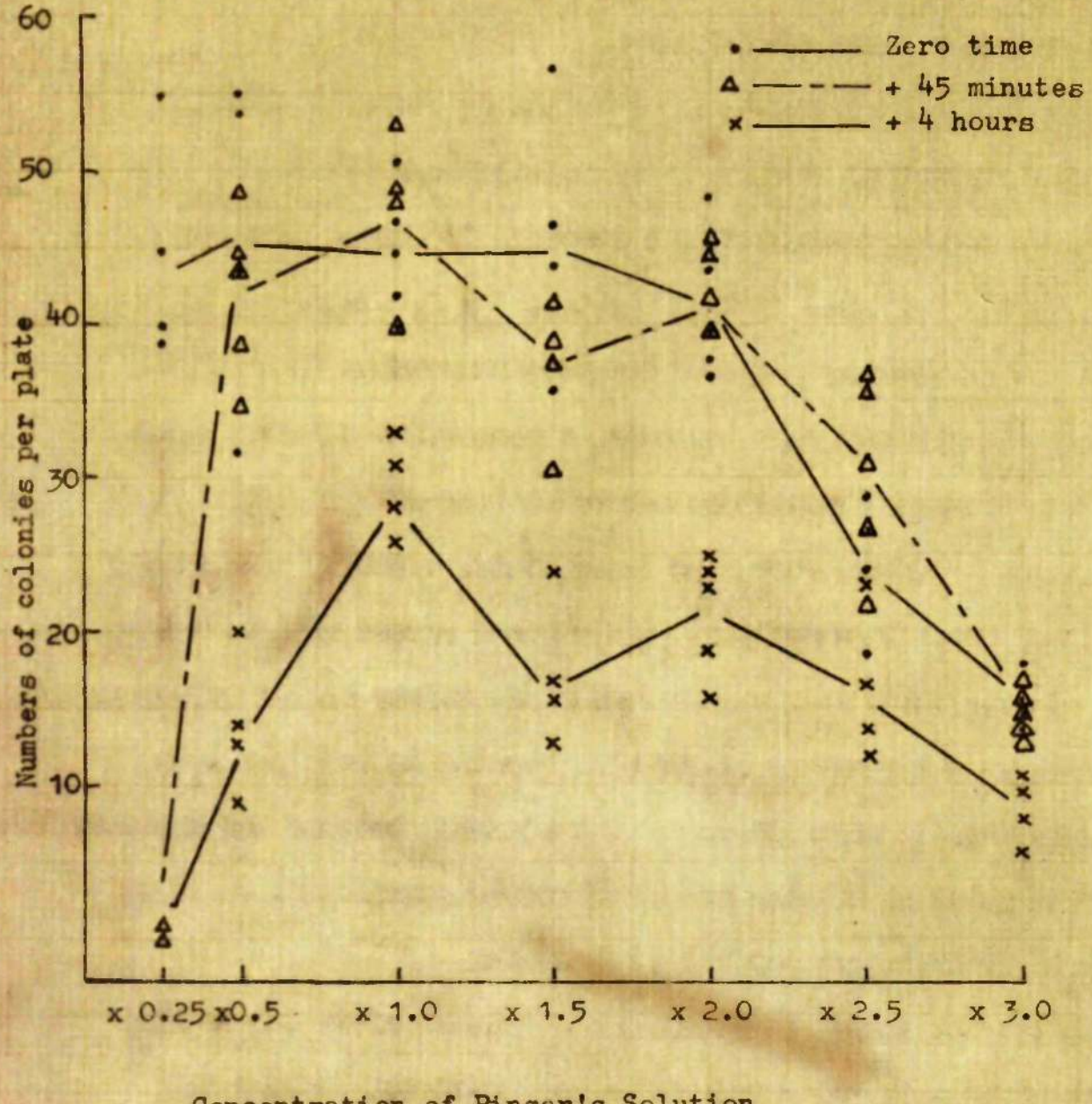


Fig.4 Comparison of the numbers of zoospores germinating to form colonies after immersion for different periods in different concentrations of Ringer's Salt Solution.

is not an osmotic phenomenon. Evidence from the above experiment, however, lends support to the suggestion that the damage may be due to an unfavourable ionic environment.

To determine the optimal concentration of Ringer's solution for use as a suspending fluid a stock solution was diluted to give the following concentrations of the standard Ringer's solution.

x 0.25; x 0.5; x 1.0; x 1.5; x 2; x 3.

One ml of a freshly prepared zoospore suspension (5×10^3/ml) in de-ionised water and thus including a proportion of burst spores, was added to 9 ml of the various concentrations of salt solution in 1 oz bottles. Platings were made immediately, after 45 minutes and after four hours. Five replicate plates were spread with 0.2 ml samples of suspension from each solution and colony counts were made after 72 hours. The number of colonies counted in each plate is recorded in Fig. 4 which shows the relationship between the concentrations of the salt solution and the number of spores germinating to form colonies after different periods of immersion.

Viability of spores is considerably reduced after suspension for a few minutes in the x 2.5 and x 3.0 solutions. Spores held in x 0.25, x 2.5 and x 3.0 solutions are significantly damaged after 45 minutes and after 4 hours spores in all concentrations of Ringer's solution have reduced viability. The highest percentage obtained after storage for 4 hours is found in samples of spores which had been suspended in the x 1.0 concentration. At least some of the

damage recorded in other concentrations of Ringer's solution could have been osmotic in origin as the osmotic pressure of the solutions used ranges from 0.47 atmospheres in the x 0.25 solution to 5.64 atmospheres in the x 3.0 solution. The most favourable concentration of sucrose and Ringer's solution for spore survival have an osmotic pressure of 1.5 atmospheres, and so it is possible that any solution with an osmotic pressure differing widely from that value will tend to damage zoospores.

It was found that Ringer's solution could not be used in the same way as sucrose to preserve zoospores during their release in strip cultures. Addition of salt solution to the de-ionised water as soon as the first zoospores were observed inhibited further zoospore release. A solution to the problem would be to remove spores in 2% sucrose and to add enough concentrated Ringer's solution to ensure optimal survival. To find the amount of salt solution required to prevent damage, the survival of zoospores was examined in 2% sucrose containing various concentrations of Ringer's salts. One-ounce bottles were prepared containing 9 ml of different concentrations of Ringer's salt solution made up with 2% sucrose. The salt concentrations were prepared such that when 1 ml of 2% sucrose, containing zoospores, was added to make a total of 10 ml, the final concentrations were equivalent to x 0.0001; x 0.001 - x 0.1 and x 1.0 of the standard concentration. 1 ml of a zoospore suspension (3.5×10^3/ml) in 2% sucrose was added to 9 ml of the various solutions and to 9 ml of 2%

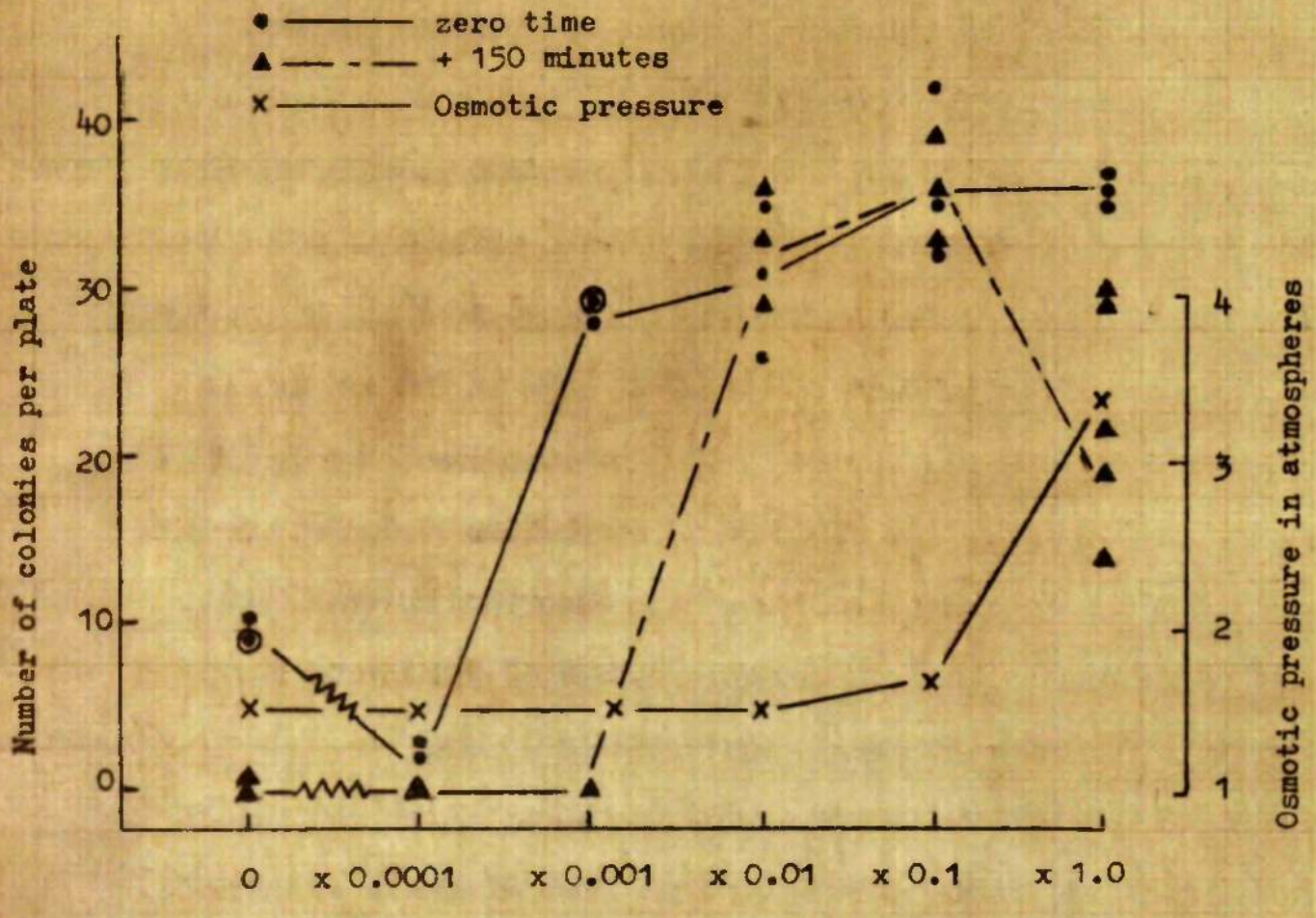


Fig.5 The relationship between zoospore viability, the concentration of Ringer's Salt Solution in 2% Sucrose and the osmotic pressure.

sucrose for a control. Three 0.2 ml samples from each treatment were plated immediately and after 2½ hours. Colony counts were recorded after 72 hours. The number of colonies counted in each plate is recorded in Fig. 5 which shows the relationship between the viability, the concentration of Ringer's solution and the osmotic pressures.

By inspections of the data in Fig. 5 it can be seen that the viability of spores suspended in pure 2% sucrose and in sucrose + 0.0001 Ringer's solution for a few minutes before plating was significantly lower than that of those suspended in sucrose containing a higher proportion of salts. More prolonged immersions resulted in poorer survival when the proportion of Ringer's salts in the sucrose fell below x 0.01 or rose above x 0.1. At salt concentrations equivalent to x 0.01 and x 0.1 of the standard concentration viability was not disturbed after 2½ hours immersion. The decreased survival below x 0.01 would seem to reflect the requirement of the spores for an environment with a higher ionic concentration. While at salt concentrations above x 0.1 the osmotic pressure will be rising beyond the limits allowing good survival. The osmotic pressure of the 2% sucrose in x 1.0 Ringer's salt solution will have a value of 3.35 atmospheres.

Thus, when spores have to remain in suspension for more than a few minutes, it is essential to use a suitable suspending solution which will protect the spores against osmotic damage or injury, possibly to the zoospore membrane, caused by a toxic environment. The above experiments have shown that a simple solution satisfying these requirements

is provided by a solution containing 2% sucrose and a concentration of Ringer's Salts equivalent to 0.1 of the standard Ringer's Salt concentration. Such a "sucrose-salt" solution would then contain the following:

NaCl	0.215 gm	$CaCl_2$	0.012 gm
KCl	0.0075 gm	Sucrose	20 gm
$Na_2S_2O_3\ 5H_2O$	0.05 gm	De-ionised water	1000 ml

A method of producing zoospore suspensions with optimal viability

The method originally adopted has been modified in the light of the above experimental results. The procedure is as follows:

1) Transfer a freshly incubated strip culture, prepared as on page , to 15°C and flood with 5 ml of de-ionised water at 15°C.

2) Add 2.5 ml of 4% sucrose at 15°C after 30 minutes.

3) Add another 2.5 ml of 4% sucrose at 15°C after a total of 45 minutes.

4) Remove the culture to room temperature (18 - 22°C) and harvest the first suspension.

5) A "second suspension" can be obtained within 5 minutes by adding 10 ml of 2% sucrose at room temperature.

6) Add 0.1 ml of x 10 Ringer's Solution to 10 ml of spore suspension. This step may be omitted when dealing with

concentrated spore suspensions (>10^4/ml) over short periods of time.

7) Dilutions are made into the "sucrose-salt" solution.

Suspensions so produced will contain a minimum of burst and damaged spores and may be left for a few hours at room temperature or for a few days at 3°C before they are added to medium. For example, a 1.6 x 10^5/ml zoospore suspension in "sucrose-salt" solution was stored for 30 hours at 3°C. Examinations showed the following spore composition: (1) 1% of the spores were still motile: (2) 6% had encysted: (3) 9% had short germ tubes, and (4) 28% had suffered obvious damage. Three replicate plates of S.M.A. were spread with 0.1 ml of a 1/100 dilution of this suspension after 30 hours, 54 hours and 78 hours storage at 3°C. Colony counts are recorded in Table 7.

Storage Time in Hours	Number of colonies per plate			
	Replicates			Total
	1	2	3	
30	19	21	17	57
54	18	22	24	64
78	23	20	18	61

Table 7. Numbers of colonies in plates spread with suspension stored for various lengths of time at 3°C.

The percentage germination, around 20% after 30 hours storage does not fall significantly during the next 48 hours of storage at 3°C.

A method of producing small volumes of zoospore suspension

A modified technique can be used when small volumes (up to 1 ml) of concentrated zoospore suspension are required. Small discs of medium approximately 4 mm in diameter and 8 mm thick are cut from a pea-meal agar plate with a cork borer and are transferred to the lid of a sterile plate. The discs are inoculated, each with a different isolate and sterile water is added to the base of the plate to prevent the discs of medium from drying. The plate is incubated for 3 days when each disc is transferred to a one-ounce bottle containing approximately 10 ml of non-nutrient agar. Bottles are incubated for 3 days; then zoospores are induced as follows:- 0.5 ml of de-ionised water at 15°C is placed in each bottle and after 30 minutes 0.25 ml of 4% sucrose is added followed by another 0.25 ml after a total of 45 minutes. The resulting 1 ml of spore suspension contains up to 10^5 zoospores.

This technique has certain advantages over the strip culture method:

1) Zoospores are produced 7 days after inoculation instead of 11 to 13 days.

2) Less vegetative growth is made prior to sporulation. This should minimise any selection acting during vegetative growth on pea-meal agar.

3) A larger number of separate asexual progenies can be sampled conveniently at the same time. The labour involved in medium preparation and culture manipulation is considerably reduced.

4) There is less chance of contamination.

A method of preparing suspensions containing zoospores of different sizes.

Zoospores produced from mycelium grown on pea-meal agar are of a uniform size, have two flagella and one nucleus. Occasionally, and especially in suspensions from old strip cultures, larger zoospores are observed which have more than one pair of flagella and more than one nucleus.

Although some sporangia are formed when strip cultures are prepared from mycelium grown on S.M.A., zoospores are rarely or never induced by the usual methods. However, if S.M.A. is supplemented with certain concentrations of cholesterol, zoospores can be induced from strip cultures. When S.M.A. is supplemented with concentrations of cholesterol between 30 and 300 µg/ml before autoclaving, the strip cultures of mycelium grown on the supplemented media bear large numbers of zoosporangia. Zoospore suspensions of up to 3×10^4 spores/ml can be induced by the usual method but the zoospores vary considerably in size. At a concentration of 10 µg/ml, cholesterol has little effect on sporulation and zoospores are rarely induced.

Cholesterol, at the higher concentrations used, stimulates the formation of zoosporangia but also interferes with the cleavage of the protoplast inside the zoosporangium during the formation of zoospores. The sizes of zoospores produced range from the smallest, which have the usual single nucleus and two flagella to the very largest multi-nucleate and multi-flagellate ones which would appear to be the complete protoplasts of zoosporangia. These must be formed when no cleavage

takes place and the whole protoplast squeezes out through the pore in the papilla. Fig. 6 shows a histogram of the numbers of spores in various size classes present in a suspension derived from a mycelium grown on S.M.A. + 30µg/ml cholesterol.

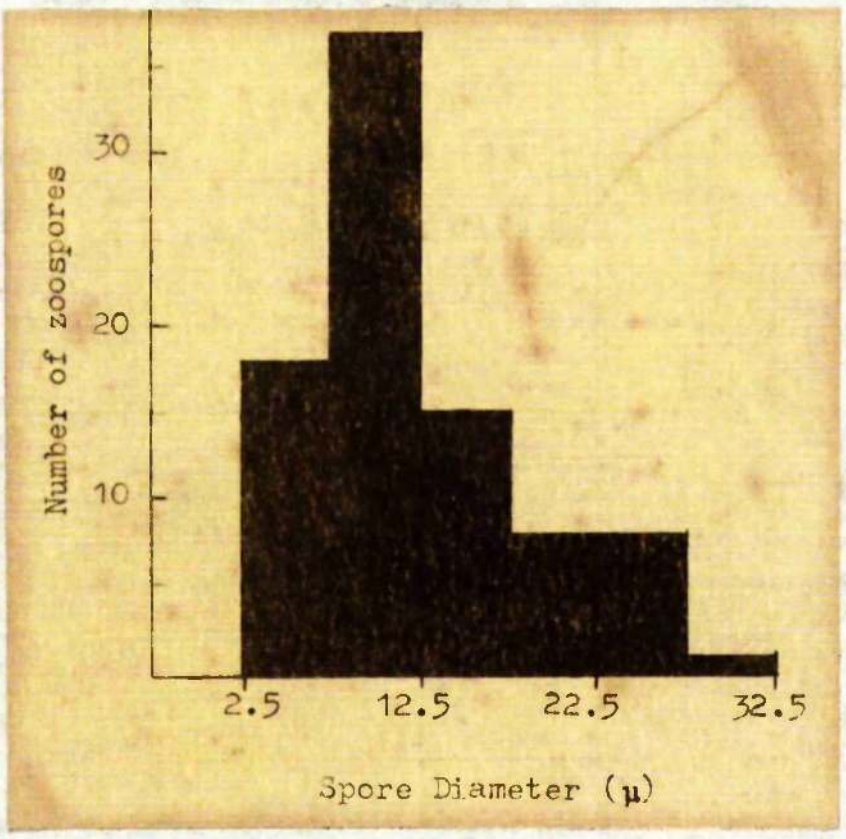


Figure 6. Histogram showing numbers of zoospores in 5µ interval size classes.

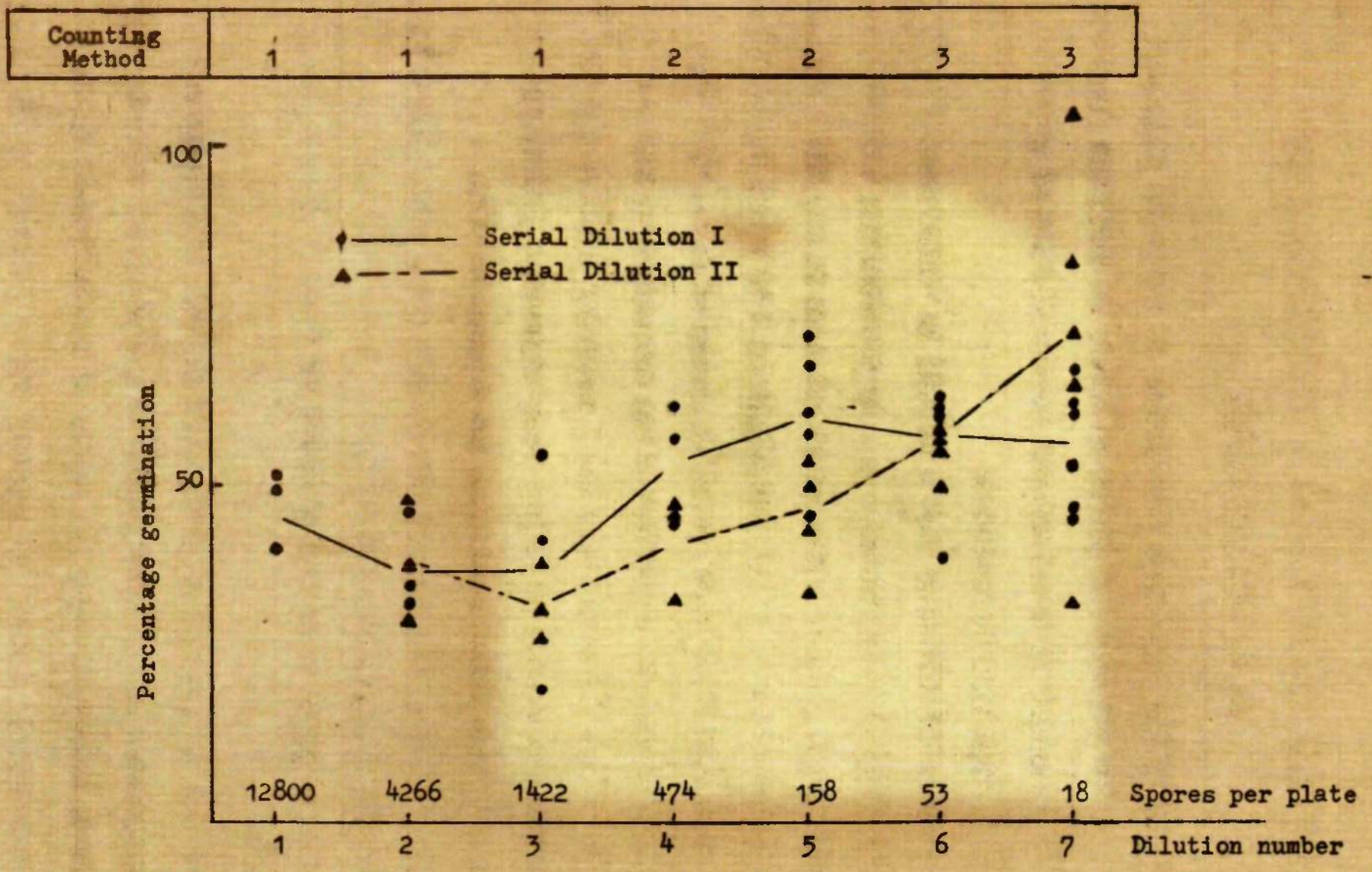


Fig.7 . The percentage germination of zoospores plated at different densities

The viability of zoospores plated on Standard Medium Agar at different densities

Estimates of the percentage germination of zoospores suspended in "sucrose-salt" solution and plated on S.M.A. at different densities were made to determine if there was any self-inhibition of spore germination in high density platings.

A suspension of zoospores (6.4×10^4/ml) in "sucrose-salt" solution was serially diluted in duplicate 1 in 3 by transferring 1 ml of spore suspension to 2 ml of "sucrose-salt" solution in one-ounce bottles. Three 0.2 ml samples were taken from dilutions 1 to 4 and five samples from dilutions 5 to 7 and were spread on plates of S.M.A. ~~(See Fig. 7).~~ The numbers of spores which germinated was determined as follows:

1) For serial dilutions 1, 2 and 3 estimates based on the number of spores germinating in 40 low power microscope fields per plate were made after incubation for 6 hours at 24°C.

2) Total counts were made of dilutions 4 and 5 with a binocular microscope after 12 hours at 24°C.

3) Colony counts were made of dilutions 6 and 7 after 72 hours at 24°C.

Values for the percentage germination in each sample were calculated on the assumption that the total number of spores spread on each plate could be calculated from the number of spores present in the original suspension. A more satisfactory way of determining the percentage germination is undoubtedly by counting numbers of germinated

and ungerminated spores but, as has already been pointed out, spores which do not germinate, frequently disintegrate completely and cannot be recognised.

The percentage germination, y, of zoospores plated at different densities, x, is shown in Table 8 and Fig. 7. In Table 9 the Analysis of Variance of the y values, transformed to angles is shown; this indicates that the regression for each dilution series is highly significant.

	Dilution Series I		Dilution Series II	
Item	Df	Mean Square	Df	Mean Square
Between Concentrations				
Regression	1	442.5	1	1090.3
Remainder	25	59.84	4	185.4
Within Concentrations	20	31.24	17	102.1

Table 9. Analysis of Variance. Percentage germination and spore concentration per plate.

The differences in germination between different concentrations of spores not accounted for by the regression are not significant compared with the differences between replicate plates within concentrations so that a straight line is an adequate description of the relation between germination percentage and spore number per plate over the range investigated. The slope of the lines calculated for the two dilution series are not significantly different as shown

		Dilution Series I		Dilution Series II	
Dilution number	Estimated number of spores plated	Estimated number germinating	Percentage germination	Estimated number germinating	Percentage germination
1	12800	5185 6455 6208	40.51 50.43 48.51	-	-
2	4266	2010 1481 1411	46.74 34.44 32.81	1622 2045 1375	37.72 47.58 31.98
3	1422	282 776 600	19.67 54.14 41.86	564 459 388	39.38 32.02 27.07
4	474	214 275 292	44.86 57.65 61.21	221 218 194	46.33 45.70 33.62
5	158	96 73 91 114 107	60.28 45.84 57.14 71.58 67.19	91 55 73 114 107	60.28 45.84 57.14 71.58 67.19
6	53	33 21 33 33 32	62.16 39.56 62.16 62.16 60.27	31 30 27 31 31	58.39 56.51 56.86 58.39 58.39
7	18	8 11 8 11 12	45.21 62.16 45.21 62.16 67.81	12 6 13 15 19	67.81 33.91 73.47 84.77 107.37

Table 8. The percentage germination at different plating densities.

Dilution Series	$S(x-x)^2$	$S(x-x)(y-y)$	$S(y-y)^2$	S. sq. for Regression	Error	Df	Mean sq. for Error
I	86.6	307.3	3568.1	1090.3	2477.8	25	
II	106.7	217.3	1366.5	442.5	924.0	21	
					3401.8	46	73.95
I + II	193.3	524.6	4934.6	1423.7	3510.9	47	
					109.1	1	109.1*

* Not significant.

Table 10. Analysis of Co-variance. To test significance of difference between the two regression coefficients.

by the analysis in Table 10, which has been carried out by the method of Goulden (1952; Chapter 9). The decreasing percentage germination in plates seeded with increasing numbers of zoospores may indicate a real inhibition of germination. It is possible that other factors such as the different methods used to estimate the percentage germination contributed to the effect. However the relationships indicated between counts made by the same method, viz. over the range of dilutions 1, 2 and 3; 4 to 5, and 6 to 7 are mostly similar to those indicated between counts made by different methods, viz. dilutions 3 to 4 and 5 to 6. (See Fig. 7).

Part III. The production of sexual progeny.

Meiotic analysis in the Oomycetes has never been carried out, due primarily to the fact that growing cultures derived from zygotes have only rarely been established. The need for the development of a technique to enable sampling of large numbers of sexual progeny is obvious.

The formation of sexual spores.

Being a homothallic fungus, single zoospore isolates of P. cactorum will reproduce sexually in a suitable environment. Until recently, certain natural media, e.g. oat-meal agar, have been used to promote oospore formation which does not take place in minimal medium agar. It has now been discovered that the oospore inducing substances in oats are sterols. Cholesterol and ergosterol stimulate growth and oospore production when used as a supplement to minimal medium at a concentration of 10µgm/ml. (Elliott et al. 1964). A medium allowing early formation of large numbers of oospores is easily prepared by adding one per cent $^{w}/_{v}$ of a light petroleum extract of oats to S.M.A. 100 gm. of crushed oats refluxed with light petroleum for one hour yields approximately 4 gm. of a yellow-brown oil. When the concentration of oat extract in the medium is lower than 1% sporangia and chlamydospores become abundant and fewer oospores are formed.

At first, plates were inoculated centrally with discs of mycelium

but later, when it was desirable to obtain oospores of a more uniform age, plates were seeded with a suspension of sporangia and hyphal fragments prepared in a tissue grinder. Plates were incubated at 24°C in the dark as growth is quickly arrested when cultures are kept in the light. Oogonia are abundant in seeded cultures incubated for 60 hours and thick walled oospores can be found after 84 hours.

The germination of oospores.

So far, attempts to germinate large numbers of oospores of oomycete fungi in pure culture have met with little success. Typically, after fertilization and after a thick wall has been deposited, the spore passes into a period of dormancy when it will not germinate under conditions which favour growth from hyphae, sporangia or zoospores. (Blackwell 1943b). This resting phase has been compared with the after-ripening period found in many angiosperm seeds. McKay (1937) reported that oospores of Peronospora destructor may remain in soil in a dormant state for as long as 4 years before any germination takes place, after which time more and more spores germinate over the following 4 years. Either the spore population in such cases was genetically heterogeneous or the period of dormancy was conditioned by environmental factors which vary during spore formation or maturation. This type of dormancy has obvious survival value especially for a pathogen whose host may not be constantly present in the vicinity.

The first report of oospore germination was by de Bary (1860) who observed germination in three-month-old spores of Aphanomyces stellatus. Other studies on zygote germination in the Saprolegniales

are reviewed by Zeigler (1948). He concludes that the length of the after-ripening period in members of this order may be as short as 30 hours or as long as 3 months. Precise observations on germination in the Peronosporales are rare but again the after-ripening period is of variable length. Pythium dictyosporium oospores germinated after storage in water for 4 months (Maciborski 1891) and Pethybridge (1914) found occasional germination in 9-month-old cultures of Phytophthora erythroseptica. Sparrow (1934) reports that newly formed oospores of Pythium angustatum germinate when placed in water or in fresh nutrient medium. Oospores of P. cactorum were found to need a ripening-period of at least 9 months (Blackwell 1943b).

The possibility of finding ways of shortening the period of dormancy in fungal spores, similar to those methods used to activate dormant seeds, has attracted a number of workers (Brown 1946). Treatments which simulate natural conditions such as alternations of temperature, variation in water content and leaching have been successful and chemicals, especially certain volatile organic compounds, auxins and vitamins have been found to activate dormant spores of some fungi (Gottlieb 1950). A different approach to the problem was provided by Machlis and Ossia (1953) who carefully regulated the cultural conditions under which spores were formed. Resistant sporangia of Allomyces germinated without a rest period when grown under conditions favouring thin spore walls.

Some of these treatments have been tried out on dormant oospores.

a) Low temperature treatments. De Bary (1881) first noted that oospores of Phytophthora omnivora, collected in June, germinated more readily in the spring of the following year after a cold December and Sparrow (1934) induced 'abundant germination' in Pythium adhaerens by storing oospores outdoors in the cold from February till April. Following up these reports Blackwell (1943b) carried out controlled experiments on the effect of temperature on germination in P. cactorum. The highest percentage germination was obtained when two to nine month old oospores were exposed for one week to a temperature just above freezing and then transferred to alkaline tap water or soil leachate. Even using this method the rate of germination was slow and total germination after a few days was only a few percent.

b) Chemical stimulation. Various extracts and decoctions have been found to induce oospore germination, but the active agents present in these are unknown. Barton (1957) observed that oospores of Pythium mamillatum germinated in the presence of turnip seedlings and cabbage water was used by Trow (1901) to germinate spores of Pythium ultimum. Savage and Gallegly (1960) tested various decoctions but also a number of pure chemical substances for activity with Phytophthora infestans. Horse dung infusion and potato soil leachate were slightly active. 2-4 dichlorophenoxy-acetic acid, 3-indole proprionic acid, coumarin, caffeine, gallic

acid and vitamin mixtures all stimulated germination but the germination recorded was rarely above 1%. A maximum of 12.5% was recorded on one occasion with coumarin.

c) Ingestion. A rather unique report in the literature (Gregg 1957) is on the germination of four to six-week-old oospores of Phytophthora erythroseptica and P. cactorum in the faeces of the land snail Helix aspersa which had been fed with cultures containing oospores. Oogonial envelopes and empty antheridia were apparently undamaged by passage through the snail gut and germ tubes bearing terminal sporangia were observed after a few days. No estimates of the percentage germination were made. It was suggested that the stimulation could be due to the action of the cellulase from the snail gut or to the activities of micro-organisms present in the gut.

The development of a technique for germinating oospores.

It was decided that the best way of tackling the problem would probably be by modifying some of the techniques which had already proved to be partly successful. To make a full investigation into

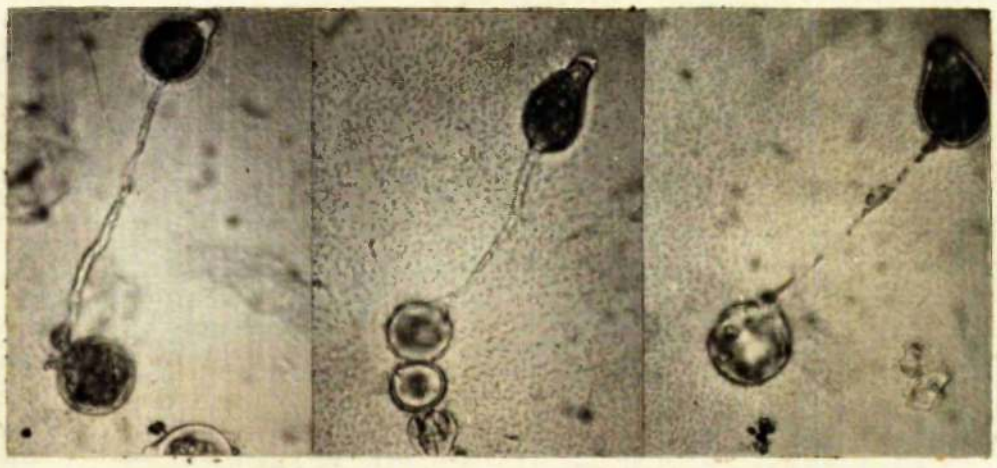


Figure 8. Oospores germinating after passage through a snail.

the physiology of oospore germination would be a major project in itself and so it was considered that too much time should not be spent on trying to find new methods of inducing germination.

Since the only report of Phytophthora oospores being germinated in any quantity within a few days of treatment was by Gregg (1957), it was decided to determine whether or not a technique could be worked out involving the use of live snails. At first, land snails (Helix aspersa and Cepaea sp.) were fed with portions of 28-day-old oospore cultures. The faeces were collected and were found to contain large numbers of oospores. The few sporangia, chlamydospores and hyphal fragments which had survived digestion proved later to be mostly inviable. Suspensions of digested oospores were washed in several changes of sterile water and were plated on non-nutrient agar (8.0 gm. "Oxoid" No. 3 agar/1 litre de-ionised water). After four days many of the oospores, now divested of their oogonial envelope, had germinated by a short germ tube with a terminal sporangium (Fig. 8). However, when a few of these germinated spores were transferred to S.M.A. they were very soon engulfed by rapidly multiplying yeasts and bacteria from the gut flora. Attempts were made to inhibit the contaminants selectively by including antibiotics and other toxicants in the S.M.A. e.g. Chloramphenicol, 100µg/ml; Benzyl-penicillin, 1,000µg/ml; Aminopterin, 100µg/ml and Potassium tellurite, 50µg/ml. No single drug was able to inhibit all the contaminants commonly occurring and combinations of drugs which covered the spectrum of contaminants

inhibited further growth from germinated spores. Several of the drugs seemed to be inducing sectoring of fungal colonies on agar media, therefore it was decided to discontinue this approach.

The possibility of using sterile cytase preparations was investigated. 4 gm. of freeze dried cytase (E. Gurr, Limited) was extracted with 40 ml of Sorensen's phosphate buffer ($^{M}/_{30}$; pH 5.5) in the cold and was sterilized using a Seitz filter. Oospore suspensions were prepared by grinding portions of oospore cultures in a glass tissue grinder. 0.25 ml volumes of washed oospore suspension in de-ionised water were incubated with 3 ml of the cytase extract or with a 1 in 10 dilution of the extract for periods ranging from 2 to 16 hours. Treated oospores were washed in de-ionised water and were plated on agar. After 4 days the cultures were over-run with mycelium growing from hyphal fragments or sporangia and no activation of oospores could be observed.

Further efforts to eliminate contamination from digested spores were continued. Spores which had passed through the gut of the water snail Planorbis corneus were activated as much, if not more, than those which had been fed to land snails. As the former are much more easily kept and seemed to have larger appetites for oat-extract cultures they were used for all subsequent studies. It was thought that surface sterilisation of the digested but still ungerminated oospore might prove to be a successful method. Accordingly, washed oospore suspensions prepared by grinding the faeces were treated

with various concentrations of mercuric chloride ranging from 0.05µg/ml to 50µg/ml for 5 minutes. Spores were then washed four times in sterile de-ionised water, plated on S.M.A. and incubated on the laboratory bench at room temperature (18 - 20°C) for 4 days. Contaminants were rare and localised to small areas on plates whose spores had been treated with 30µg/ml and 50µg/ml of mercuric chloride. Growth from germinating spores was rapid and soon engulfed the still ungerminated spores and it was difficult to tell whether a colony had originated from an oospore or from a stray sporangium. However, on non-nutrient agar, growth from spores is strictly limited, making it possible to follow the rate of germination and to identify the germinating spores. Typically, on non-nutrient agar the oospore produces one short germ tube with a terminal sporangium whereas the occasional sporangium or chlamydospore germinates to produce one or a few germ tubes which grow out and branch frequently.

The effect of oospore age on the percentage germination.

Plates of oat-extract agar were seeded so as to give oospore cultures 7; 14; 21 and 28 days old. Portions of each culture were fed to Planorbis. Three snails were allowed to feed on each mycelium of a different age in a beaker. Excreta, collected after 48 hours, was homogenised in a tissue grinder, the suspensions were washed in water and then treated with an equal volume of mercuric chloride (60µg/ml) for 5 minutes. Washed spores

were plated on non-nutrient agar. Four replicate plates were spread with a sample of spores from each suspension. Two replicates of each age were incubated at 24°C in an incubator and two replicates were incubated on the laboratory bench at room temperature. The percentage germination was determined by scoring 100 spores in each plate after four and eight days. The results are recorded in Table 11.

		Maximum Age of Oospores							
		4 days		11 days		18 days		25 days	
		Replicates							
		1	2	1	2	1	2	1	2
Scored after 4 days	Bench	0	0	1	0	6	9	21	20
	Incubator	0	0	0	0	0	0	0	0
Scored after 8 days	Bench	0	0	13	1	50	53	66	67
	Incubator	0	0	0	0	1	3	20	5

Table 11. Showing the percentage germination of oospores of different ages.

The differently aged cultures gave spores of a maximum age of 4, 11, 18 and 25 days respectively. It is obvious that the percentage germination increases with the age of the oospores. It is also obvious that germination is inhibited by incubation in the dark at a constant temperature of 24°C. This result suggested that oospore germination may have a light requirement.

The effect of light on oospore germination

Serokine (1876) discovered that oospores of Aphanomyces would germinate only if they remained in the light. The only other report on this light effect is that by Zeigler (1948) who found that light is necessary for the germination of oospores of Protoachlya hypogyna, Achlya recurva and Thraustotheca primoachlya. As these were the only species examined for this effect it is possible that light may be a requirement for the germination of most if not all oomycete oospores.

An experiment was designed to verify that the stimulation recorded in the previous experiment was indeed due to the effect of light. Digested oospores, 30 days old were sterilized in the usual fashion, washed and plated on 6 plates of non-nutrient agar. 3 plates were enclosed in a light proof cardboard box and all six cultures were placed in an incubator at 24°C. A 15-watt tungsten electric light bulb was fitted inside the incubator and cultures were examined 4 days later. One hundred oospores were scored for germination in each plate. The results are tabulated below.

	Replicates		
	1	2	3
Cultures in Dark	3	3	4
Cultures in Light	36	38	27

Table 12. Percentage germination of oospores kept in the light and kept in the dark.

It is obvious that light stimulates the germination of activated spores.

The method of producing single oospore cultures.

1) Twenty-one day-old cultures on oat-extract agar are fed to live snails (three snails per culture).

2) Faeces collected after 48 hours, are homogenised in a tissue grinder, the suspension is washed and treated with an equal volume of mercuric chloride solution (60µg/ml) for five minutes.

3) Surface sterilised oospores are washed in four changes of sterile de-ionised water and are plated at a density of approximately 1000 per plate on non-nutrient agar.

4) Cultures are incubated on the laboratory bench in the light for four days.

5) Individual germinated oospores are cut out on small blocks of agar and transferred to plates of S.M.A. Small single oospore colonies are formed within two days.

SECTION II

THE PRODUCTION OF NUCLEAR MARKERS - DRUG RESISTANCE

Introduction

It has already been pointed out (see General Introduction) that the position of meiosis, the ploidy of the somatic nuclei and the existence of heterokaryosis etc. can be determined by observing the segregation and recombination of mutant characters in the progeny of a strain bearing several nuclear markers. It is to be expected that, as in higher organisms, most of the genetic material forms part of polygenic systems in which the majority of single gene mutations give rise to phenotypes which are not sufficiently distinguishable from the wild-type to be useful as nuclear markers. Mutants for this purpose need to be carefully chosen.

Easily selected mutations which are most commonly used in microbial genetics are:

1) Visible mutations. These are selected by inspection and include variants differing from wild-type in pigmentation and morphology.

2) Biochemical mutations. The most widely used class of mutations are those which cause a blockage in a synthetic pathway and which result in a change from prototrophy to auxotrophy. The mutant organism will grow only if supplied with a specific metabolite not required by the wild-type. Such mutants may

be selected by a replica plating technique.

3) Adaptive mutations. Single gene changes occur which enable an organism to grow in a previously unfavourable environment. Examples of this type of mutation are reversion to prototrophy and mutation from drug sensitivity to drug resistance. Mutants are easily selected by placing large numbers of organisms in the unfavourable environment and examining the survivors for changes in genotype.

It was decided to search for nuclear markers conferring drug resistance for the following reasons:

a) Although a change in pigmentation could be a most useful nuclear marker it is hardly to be looked for in a fungus belonging to a group which produces no distinct pigments.

b) Spontaneous and induced morphological variants of P. cactorum have been isolated but from the patterns of inheritance which they exhibit (Section III) it is unlikely that the changed phenotypes resulted from mutations of single nuclear genes.

c) Auxotrophic mutants so widely used in current genetical research, have not been found in Phytophthora spp. Recent attempts to isolate them in P. cactorum (Buddenhagen 1958) and in P. infestans (Clarke 1964) have not been successful. This may be because the nuclei are diploid - see General Introduction.

d) Certain mutations to drug resistance in fungi have been

shown to be dominant or semi-dominant in heterozygous diploid nuclei. e.g. Acriflavin resistance in Aspergillus nidulans (Roper & Käfer 1957) and acti-dione resistance in diploid yeast (Middlekauff et al 1957). On the assumption that somatic nuclei of P. cactorum could be haploid, diploid or polyploid it seemed reasonable to concentrate efforts on the production of drug resistant mutants, some of which could be dominant or semi-dominant

Origins of Resistance to Toxic Agents - Review of Literature

The intensive study of toxic agents which followed Ehrlich's discovery of the "magic bullet" has resulted in the accumulation of a vast literature on the selectivity, mode of action and clinical uses of drugs. Early in the study of chemotherapy it was realised that organisms at first sensitive to a drug, were able to adapt themselves to grow in the presence of that drug. The nature of changes in tolerance of organisms to drugs has occupied biologists for many years and still poses many unsolved problems. As the resistance of bacteria to selective agents is of great practical importance in medicine, most investigations of drug resistance have been carried out with them.

For many years there were two conflicting points of view as to the nature of the change which conferred resistance on formerly sensitive organisms. Some workers believed that resistance originated only

by gene mutation, while others thought that only phenotypic adaptation occurred; without change in the genetic material. It is now generally agreed that either or both types of adaptation can be responsible for increased drug tolerance.

Resistance due to changes of the chromosomal material.

With the development of techniques allowing genetical analyses of certain strains of bacteria (Lederberg 1947) it was shown that streptomycin resistance/dependence/sensitivity of Escherichia coli was determined by alleles at a single chromosomal locus.(Demerec 1951). Many other reports of single gene mutation to resistance to a variety of drugs have followed. It has been shown that mutation to resistance may be spontaneous and that the drug merely selects organisms which are already resistant, e.g. by "fluctuation analysis" (Luria & Delbrück 1943; Demerec 1948) and respreading techniques (Newcombe 1949). Certain drugs are known to be non-specific mutagens, e.g. manganous chloride (Demerec & Hanson 1951), acridine dyes (DeMars 1953) and several base analogues and antibiotics (Szybalski 1958) but there is no evidence to suggest that these drugs induce specific changes to resistance.

In crossing studies with bacteria it has been shown that resistance to certain drugs can be conferred by genes at a number of loci. e.g. terramycin and chloramphenicol resistance in E. coli. It seems that successive gene mutations result in the development of successively higher levels of resistance (Cavalli and Lederberg 1953). Partial resistance of E. coli to streptomycin may also be polygenic in origin

Resistance which originates in this way shows a multi-step pattern; i.e. during sub-culture on drugged medium, periodic tests show that the level of drug tolerated may increase by a small amount on a number of successive occasions, e.g. Penicillin resistance of Staphylococcus (Demerec 1948). This contrasts with the one step pattern of resistance characteristic of strains in which a single mutation confers full resistance. e.g. resistance of E. coli to azide (Lederberg 1950).

Resistance of fungi to toxic agents has not been studied so intensively. Representative plant pathogenic fungi have been screened against large numbers of chemicals in a search for suitable fungicides but references to changes in tolerance have rarely been made. With the increased interest in fungal genetics, more intensive searches have been made for mutations to resistance for use as nuclear markers. One of the first studies of resistance was made by Emerson and Cushing (1946) who showed that resistance to sulphanilamide and dependence on this anti-metabolite in Neurospora crassa were determined by single genes. Many other reports of single gene mutations to resistance have appeared since. (Howe & Terry 1962; Hsu 1962; Middlekauff et al. 1957; Lindegren et al. 1959; Morpurgo 1962; Mastie 1960 and others). The complex segregation patterns found in certain crosses involving strains of Neurospora crassa sensitive and resistant to canavanine (Horowitz & Srb 1948) suggests that multiple genes may be determining resistance in these cases.

The frequency of mutant drug resistant phenotypes in sensitive populations has been recorded for many bacteria and some fungi.

When it is known that resistance may not always arise by single gene mutations, estimates of frequencies of true gene mutations may in some cases be open to serious objection. The frequency of resistant phenotypes has been shown to vary with the chemical composition of the environment (Rosanoff and Sevag 1951). It is often not clear whether these differences in mutant frequency result from different rates of mutation or from inability of certain mutant phenotypes to be expressed under certain cultural conditions. In E. coli mutation to streptomycin resistance has a frequency of 1×10^{-9} per cell division (Demerec 1951). Resistance to streptomycin and isoniazide in Mycobacterium ranae is obtained with a frequency of 1×10^{-6} and 1×10^{-9} per cell division respectively (Hsie & Bryson 1950; Szybalski & Bryson 1952). Gene mutation to streptomycin resistance in Chlamydomonas has a frequency of 1×10^{-6} per cell division (Sager 1962). Roper & Käfer (1957) report that spontaneous resistance to acriflavin in Aspergillus nidulans occurs with a frequency of between 5×10^{-6} and 1×10^{-8} per conidium. The frequency of mutant phenotypes resistant to acriflavin in Neurospora crassa induced by ultraviolet irradiation (99% kill) was estimated to be 1.6×10^{-6} per viable spore (Howe & Terry 1962).

Resistance not due to changes in the chromosomal material.

Many examples of increased tolerance to toxic agents have been found which cannot be correlated with chromosomal gene changes. Sinae and Yudkin (1959a) have shown that some of the increased tolerance of Aerobacter aerogenes to proflavine could be accounted for by increasing

acidity in the medium during growth. They also showed that cycles of increasing and decreasing resistance are apparent when E. coli is grown in partially synchronised culture with proflavine (Sinae & Yudkin 1959b). This could account for the range of tolerance found in cultures of sensitive organisms. This work shows that care must be taken in the interpretation of data which indicates the occurrence of adaptive changes.

However many cases have been reported where loss of sensitivity can be related to extra-chromosomal change. Adaptive cytoplasmic changes, or phenotypic as distinct from genotypic adaptations, may be specifically induced by the drug, often at a high rate. A well known example is the induction of penicillinase by penicillin, allowing complete populations of Bacillus cereus to become resistant to the drug (Pollock 1953). A cytoplasmic state conferring greater drug tolerance may be selected during growth with sub-inhibitory concentrations of drug. Jinks (1959a) demonstrated that it is possible to select for increased tolerance to mercuric chloride in Aspergillus glaucus, even although organisms in the direct selection line are never exposed to the toxicant. These changes were shown to be cytoplasmic by heterokaryon tests.

Organisms which have become adapted to a toxic environment may retain increased tolerance in drug-free media or may become de-adapted sooner or later. The stability of the increased tolerance may be indicative of the type of extra-chromosomal change which has occurred.

within the cell. In some examples of induced enzyme synthesis the ability to produce the enzyme is lost immediately on the removal of the inducer, e.g. β-galactosidase synthesis in E. coli (Rickenberg et al. 1953). In the case of penicillinase synthesis by Bacillus cereus, however, the enzyme continues to be produced in the absence of the inducer. The ability to synthesise the enzyme persists for many cell generations until it is "diluted out" during growth (Pollock 1958). The presumed phenotypic adaptation of Aerobacter aerogenes to proflavine varies in its persistence. Cells which have only newly acquired resistance to the drug lose it readily in drug-free media. As training proceeds, reversion takes place less readily until the adaptation appears to be stable but is never absolute (Davies et al. 1944; Dean and Hinshelwood 1954). Jollos (1921) increased the tolerance of strains of Paramecium caudatum to arsenious acid by sub-culture in increasingly toxic media. When resistant clones were cultured in arsenic-free media tolerance sometimes persisted through as many as 600 cell fissions. This semi-permanent adaptation, of cytoplasmic origin, was termed "dauer-modifikation". The permanent cytoplasmic changes conferring streptomycin resistance on Chlamydomonas are thought to be "mutations" of self reproducing particles or "non-chromosomal genes" (Sager & Yoshihiro 1961).

Where it is not possible to demonstrate changes of genotype in adapted organisms it is obvious that the ability to adapt by cytoplasmic change is ultimately conditioned by the genotype. Strains of the

same organism often differ in their ability to adapt. Jinks (1959a) showed that differences in the level to which different homokaryon clones of Aspergillus glaucus could become adapted to mercuric chloride were nuclear in origin.

In Bacillus cereus gene mutations have been observed which affect penicillin resistance level, the rate of formation of penicillinase and the inducibility of enzyme synthesis by penicillin (Pollock 1959). There is no reason to believe that both genotypic and phenotypic adaptation could not occur simultaneously or in sequence in the development of resistance.

Substances inhibitory to the growth of Oosycete fungi.

These organisms have rarely been included in screening programmes with toxic chemicals. A number of isolated reports have referred to substances inhibiting members of the Peronosporales. Uppal (1926) tested a number of organic compounds including organic acids, aldehydes and alcohols for their ability to inhibit spore germination in Phytophthora colocasiae. The growth of species of Phytophthora was found to be differentially sensitive to small quantities of malachite green in the culture medium (Leonian 1930). Streptomycin has been used experimentally as a systemic fungicide to control Pseudoperonospora

on hop (Horner & Maier 1957). Vaartaga (1960) found that of a large number of fungicides tested only thiram, acti-dione, thuyaplicin, aureomycin and ethylsulfonylethylene (C-272) were toxic to P. cactorum in low doses. Other antibiotics found to be inhibitory to Phytophthora spp. were novobiocin, patulin, oxytetracyclin and tetracyclin (Ackert & Tsao 1962).

Drug	Source	Minimal inhibitory level preventing colony formation from zoospores (µg/ml)
acti-dione (cycloheximide)	Light & Co., Ltd.	10
antimycin	----	>250
benzyl penicillin	Glaxo Labs., Ltd.	>1000
chloromycetin succinate	Parke, Davies & Co.	1000
chlortetracyclin	Pfizer, Ltd.	100
patulin	London School of Hygiene & Tropical Medicine	1.0
polymyxin	Glaxo Labs., Ltd.	100
streptomycin sulphate	Glaxo Labs., Ltd.	100
tetracyclin	Pfizer, Ltd.	100
8-azaguanine	Light & Co., Ltd.	>1000
5-bromouracil	Light & Co., Ltd.	>1000
aminopterin (amino pteroylglutamic acid)	----	100
sulphanilamide	B.D.H., Ltd.	3440
D-L ethionine	Light & Co., Ltd.	>1000
p-fluorophenylalanine	Light & Co., Ltd.	100
acriflavin	Hopkin & Williams, Ltd.	100
malachite green	Hopkin & Williams, Ltd.	0.3
potassium tellurite	Hopkin & Williams, Ltd.	100

Table 13. Substances screened against P. cactorum.

The Search for Drug Resistance in P. cactorum

The selection of suitable toxic agents.

Eighteen substances, including common antibiotics and anti-metabolites were screened against the fungus (Table 13). The criteria used to determine inhibition were:

1) The ability to inhibit the growth of the fungal colony from a disc inoculum of hyphae.

2) Ability to inhibit colony growth from zoospores plated on agar.

Drugs were added at various concentrations to S.M.A. Sterile stock solutions were prepared in one-ounce bottles and suitable volumes were added to molten S.M.A. after autoclaving and just before plates were poured. Drugs which were sparingly soluble in water (8-azaguanine, chlortetracyclin and 5-bromouracil) were dissolved in dilute hydrochloric acid. In such cases the amounts of acid in each concentration of drugged medium and in the control plates was brought to the same level.

1) Inhibition of colony growth. The relationship between rate of increase of colony diameter (linear growth rate) and drug concentration in the medium was determined. 1 mm discs taken from the edge of an actively growing colony on S.M.A. were used as inocula. At least three replicate plates were incubated at 24°C. At least four measurements of colony diameter were made at two to three day intervals; two diameters

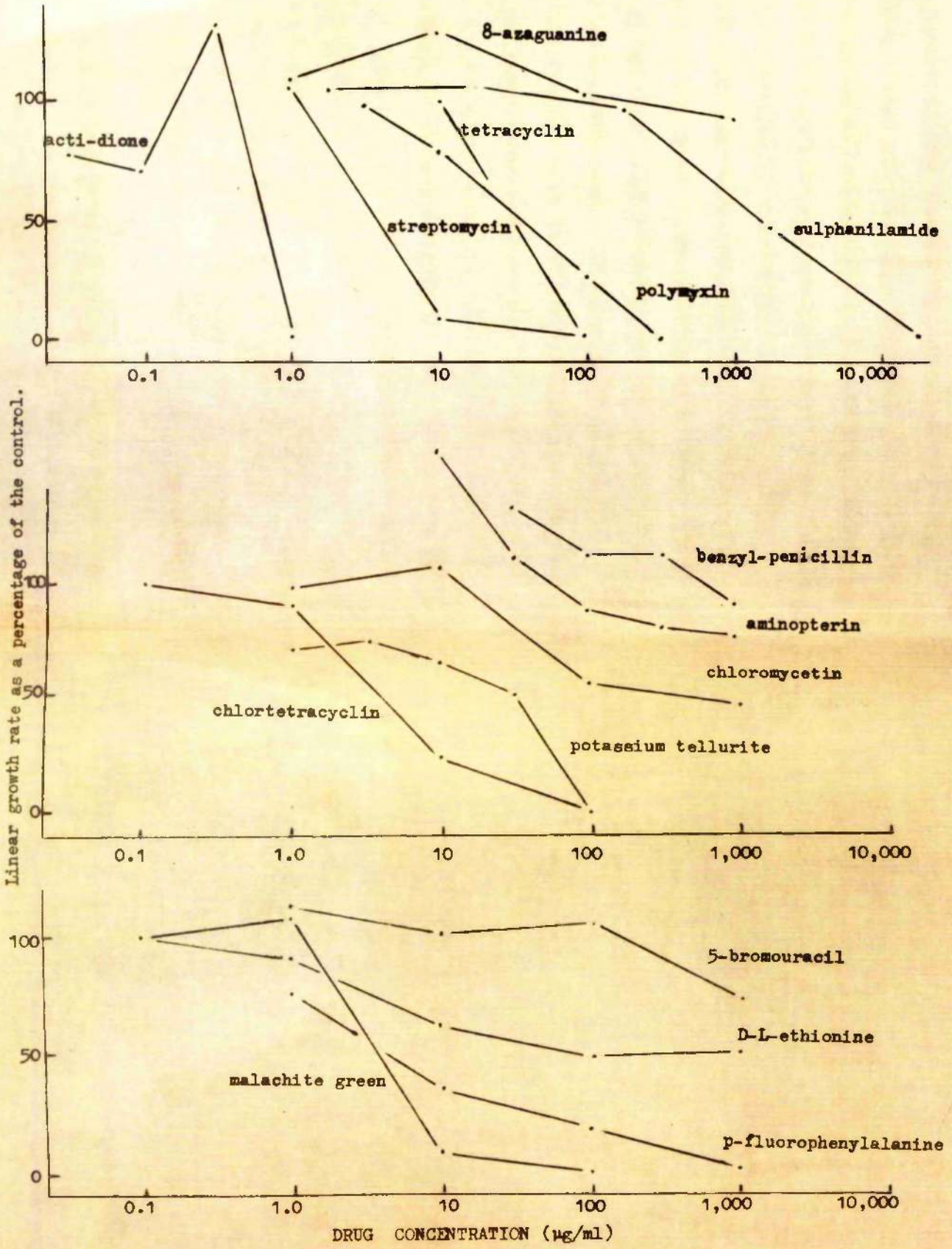


Figure 8. Dosage response of P. cactorum to various toxicants.

of each colony was noted each time. The mean diameter was plotted against time for each concentration and the linear growth rate in mm/24 hours was estimated from the graph. The growth rate on drugged plates was then expressed as a percentage of the rate on the control plates and was plotted against drug concentrations. This was done to standardise the growth rate of the controls which was found to vary between different experiments. Figure 8 shows the responses of linear growth rate to different concentrations of various drugs. It should be stressed that measurements of linear growth rates do not take into account differences in colony density which occur at different concentrations of some drugs. The increased linear growth rate at sub-inhibitory concentrations of acti-dione only indicates the rapid extension of a sparse mycelium. It can be seen that a number of the drugs are efficient inhibitors of vegetative growth under these experimental conditions but concentrations completely inhibiting growth vary considerably. Some of the base and vitamen analogues and penicillin seem to stimulate growth particularly at the lower concentrations tested. With some inhibitors, e.g. tetracyclin and acti-dione, mycelial growth was found to occur after a lag period of more than 10 days at concentrations which initially prevented any linear growth.

2) Inhibition of colony establishment from zoospores. Approximately 6×10^3 zoospores (0.2 ml of suspension) was spread on each of 3 replicate plates at each drug concentration. The minimum concentration of drug which prevented the establishment of macroscopic colonies after incubation for 72 hours was noted. The minimal inhibitory

concentrations for the various drugs are recorded in Table 13. At drug concentrations which inhibit mycelial growth, short germ tubes may be produced by zoospores, e.g. germ tubes up to 50 μ long are produced by zoospores plated on 100 μg/ml streptomycin, but further extension is prevented. At high concentrations of p-fluoro-phenylalanine and tetracyclin germ tubes from zoospores continue to grow slowly so that a microscopic mycelium is formed after prolonged incubation.

The following drugs appeared to be suitable for use as selective agents: sulphanilamide; p-fluorophenylalanine; acti-dione; patulin; streptomycin; tetracyclin; malachite green. Potassium tellurite was rejected because of its apparent effect in inducing changes in growth rate and morphology.

The Selection of Resistant Strains

Emerson and Cushing (1946) grew Neurospora crassa in growth tubes of media containing sub-inhibitory concentrations of sulphanilamide. After prolonged sub-culture a strain was produced which had a shortened lag period and an increased drug tolerance later shown to be under gene control. The method adopted by Roper and Käfer (1957), Rowe and Terry (1962) and others was simply to plate large numbers of wild-type asexual spores on agar media containing enough drug to inhibit the growth of wild-type colonies. Resistant colonies which appeared were isolated and their tolerance compared with the wild-type.

Three methods have been used to select spontaneous resistant mutants in the present investigation.

1) The plate spreading method: Between 10^3 and 10^4 zoospores (0.2 ml of a suspension) were spread on plates of S.M.A. containing inhibitory concentrations of drug.

2) The liquid culture method: 5 ml of a concentrated spore suspension containing between 5×10^4 and 5×10^5 zoospores were added to two-litre flasks containing 500 ml of the selective medium. This method is less laborious than the first and thus allows greater numbers of spores to be screened.

3) The sandwich plate method: Plates containing a thin layer of drug-free medium were spread with approximately 10^4 zoospores and incubated for 24 hours at 24°C. 15 ml of molten selective medium at 37°C was then added to each plate. Growths which appeared on the surface after further incubation were tested for increased tolerance. This method enables much larger numbers of nuclei to be exposed to the drug than is otherwise possible using ungerminated spores.

Attempts were made to induce mutation to drug resistance by the use of ultraviolet irradiation. In determining the sensitivity of bacterial cells or fungal spores to ultraviolet light (UV), the usual method adopted is to irradiate a suspension of the cells for varying lengths of time and to spread them on agar media. Viable counts

are made for bacteria and compared with similar counts on control plates.(Demerec & Latarjet 1946). Owing to the larger size of colonies formed by fungi, it is usually necessary to dilute irradiated suspensions so that colony counts are made from plates containing different numbers of spores (Hollaender et al. 1945 and others).

A "Camag" 8W UV lamp with a maximum output at 2537Å was used to irradiate suspensions at a lamp to subject distance of 20 cms. 3 ml samples of a concentrated zoospore suspension in "sucrose-salt" solution were dispensed into sterile plates and irradiated for various lengths of time with constant agitation. Irradiation and incubation was carried out in a darkroom using a yellow safelight where necessary.

At first, concentrated zoospore suspensions were irradiated for various times and then diluted 1 in 10 and 1 in 100. Two sets of plates were spread with 0.2 ml samples of suspension and colony counts were made after 72 hours. The numbers of colonies arising in plates spread with the 1 in 10 dilution after short periods of irradiation were too high for counts to be made. Also platings from the 1 in 100 dilution gave rise to convenient numbers of colonies for counting but colonies became exceedingly rare, of course, when suspensions had been irradiated for longer periods. Where it was possible to make counts in both sets of plates spread with dilutions of the same suspension, it was found that plates spread with the 1 in 10 dilution often contained only five times the number appearing in plates spread with the 1 in 100 dilution, although precautions were taken to prevent error due to

inaccurate dilution. It appeared that viability was being influenced by plating density. An inhibition of zoospore germination was noted at high plating densities in Section I, part II and the reconstruction experiment reported later in this section showed how drug inactivated spores inhibit the growth of resistant ones. The presence of dead or inactivated spores seems to inhibit germination of viable spores especially in high density platings.

Attempts were then made to determine the percentage germination of zoospores in samples diluted by the same amount. It was pointed out in Section I, part II that counts of germinated spores were difficult to make as dead spores often disintegrate. An added complication is that zoospores may germinate by producing a short germ tube but fail to make any further growth. Also after UV treatment spores may produce a germ tube very slowly and only form colonies after a considerable lag period. It was therefore decided to score as germinated those spores which had produced a germ tube longer than 300 μ after 18 hours incubation. Most germ tubes of spores from the control suspension are then at least twice that length and are considerably branched.

Samples of a zoospore suspension (5×10^4 spores/ml) in "sucrose-salt" solution were irradiated for 0, 2, 4, 8, 16 and 24 seconds. 3 ml of liquid standard medium was added to each plate receiving a different treatment and plates were incubated for 18 hours. Five-hundred spores were scored for germination in each plate and the fraction surviving after each treatment was expressed as a fraction

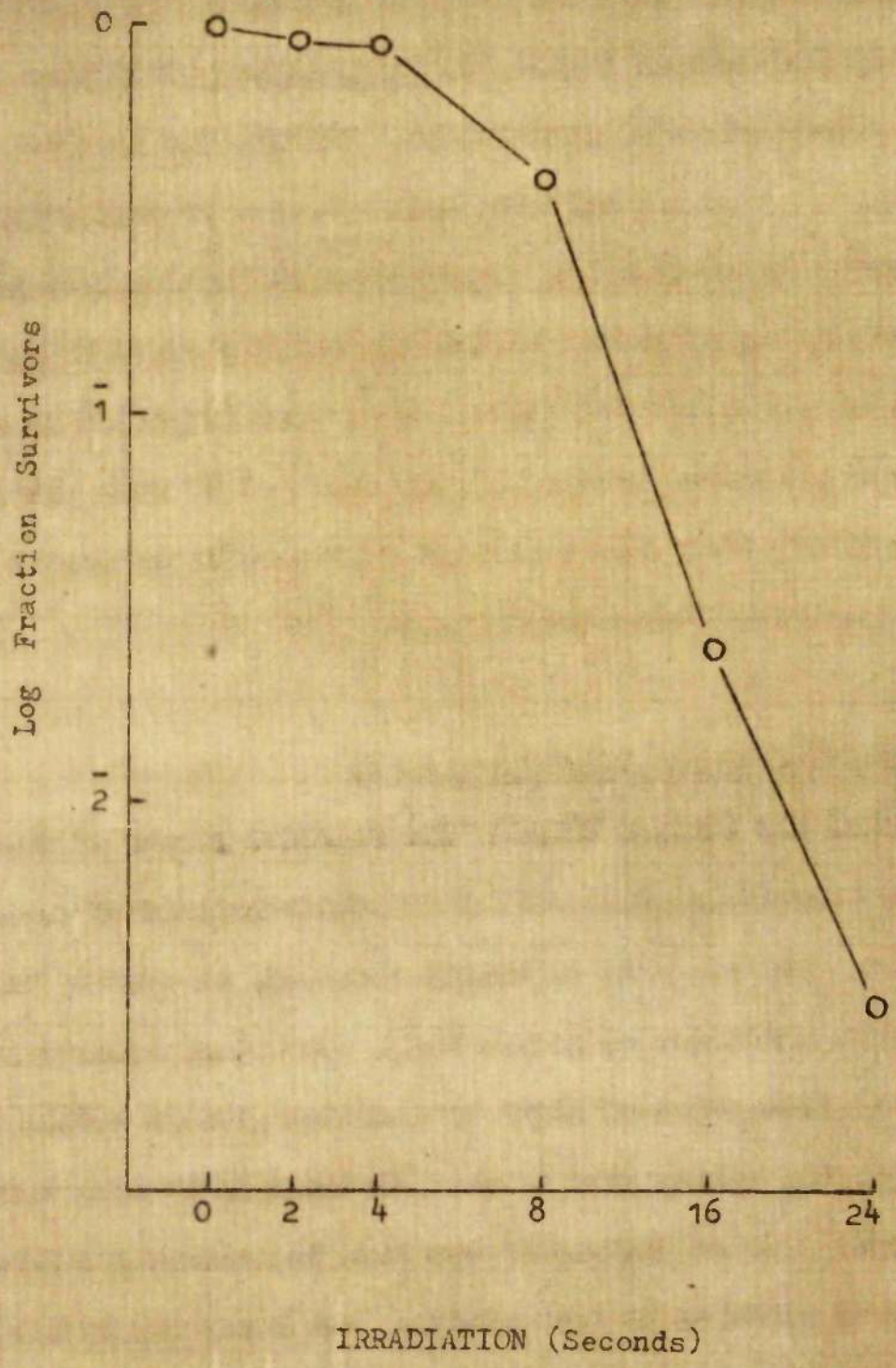


Figure 9. Inactivation of zoospores by ultraviolet irradiation

of those surviving in the control plate which was not irradiated. A killing curve is shown in Figure 9. The period of irradiation required to produce a 99% kill as determined by the above method was approximately 20 seconds. The curve obtained is similar to that obtained by Buddenhagen (1958) for P. cactorum. Both show a definite shoulder at low doses of irradiation. However the survival of Buddenhagen's zoospores may have been affected by damage sustained by suspension in water and by suppression in high density platings. In preliminary experiments with UV, the killing curve had a steeper slope but in those experiments zoospores were suspended in 2% sucrose. The suspensions which received higher doses of UV were plated last and it is likely that they contained a proportion of spores damaged by the unfavourable ionic environment.

A photometric method of measuring growth.

A method was devised whereby the relative growth of colonies growing on agar medium with different concentrations of drug could be compared. Measurements of colony diameter, as already pointed out, are poor estimates of growth where the colony density is variable. The relative absorption of light by colonies gives a better estimate of total growth, taking into account differences in both diameter and density. A Zeiss photometer was used to measure relative light absorption of colonies on agar plates. The apparatus is illustrated in Figure 10. Eyepiece, objective and condenser lenses were removed

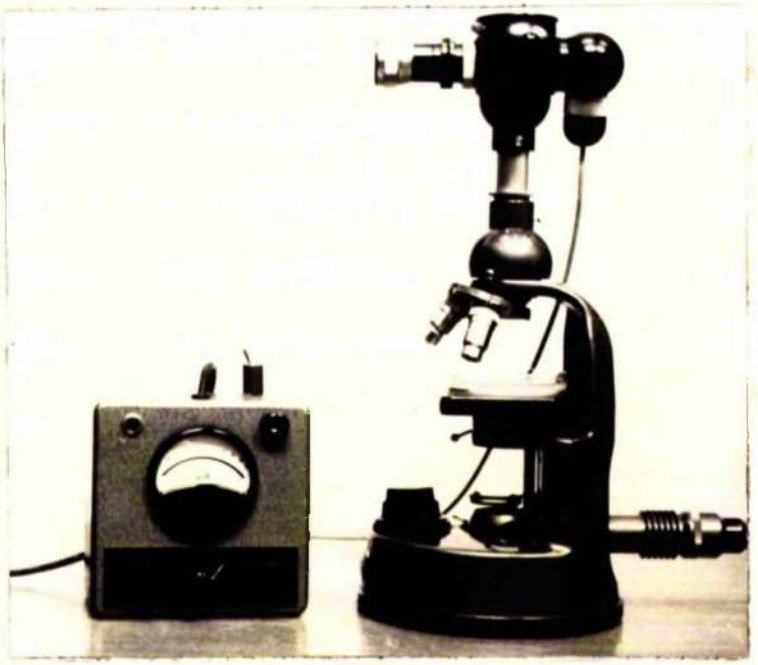


Figure 10. Apparatus used to measure relative light absorption of colonies growing on agar media.

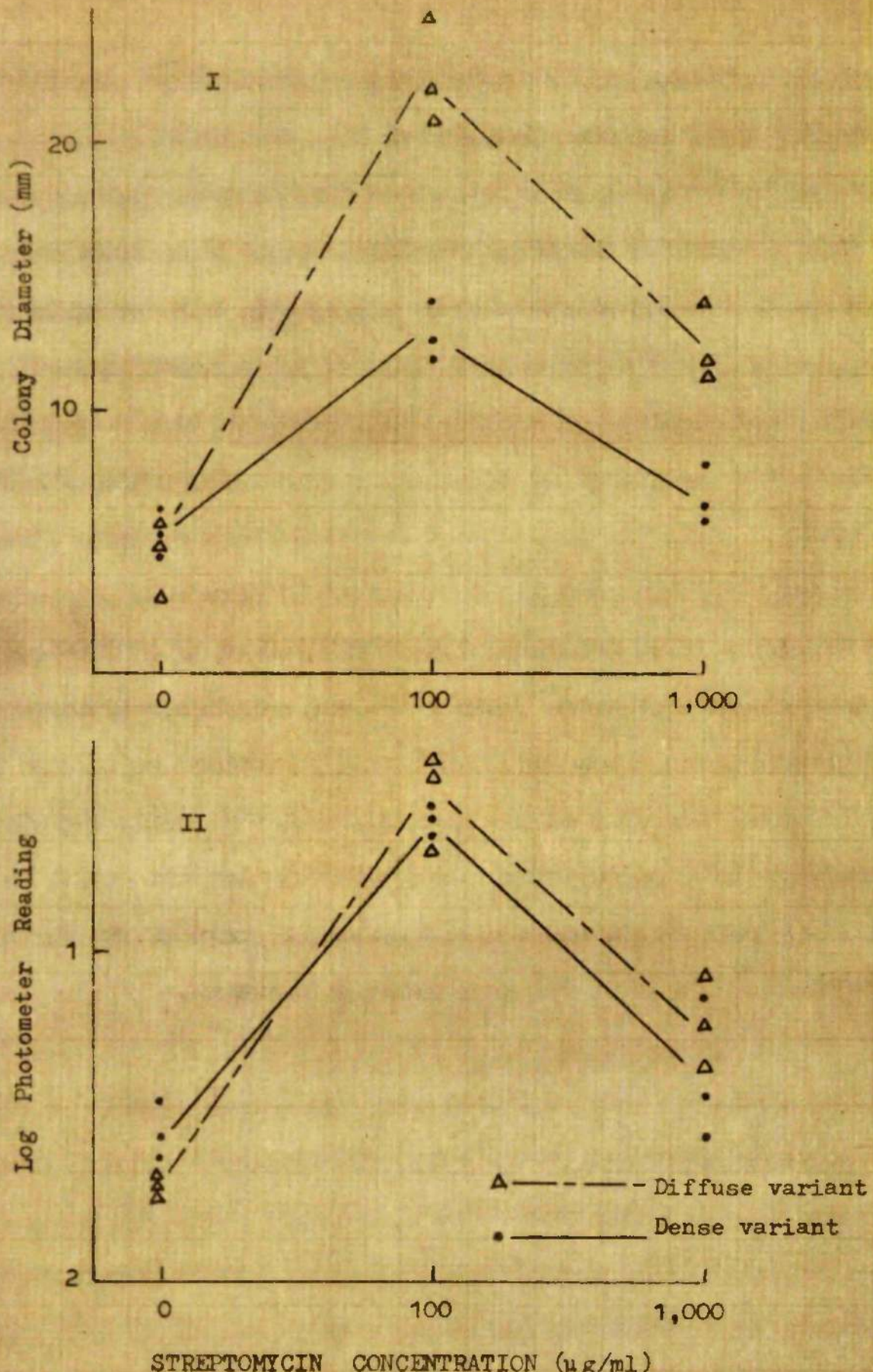


Figure 11. The dosage response of variants of Sd measured by I Colony diameter, II Photometer.

from the microscope and a diffusion screen was placed over the high intensity light source. Readings of the relative light absorbed are made on the meter which has an arithmetic scale reading from 1 to 100. To compare the relative absorption of light by colonies growing on a plate, the light intensity is adjusted so that the meter records 100 on an uncolonised portion of the plate. Relative absorption readings are obtained on centring each colony in turn over the light source. The photometer is sensitive to increasing colony diameters up to 15 mm and so it is possible to measure nine colonies growing on one plate. Error due to scratches on the bottom of the plate and variations in glass thickness could be minimised if clear disposable plastic dishes were used. Figure 11 shows the dosage response to streptomycin of a dense and a diffuse morphological variant of a streptomycin dependent strain as measured by 1) colony diameters, 2) relative light absorption. It is obvious that the former method indicates that the variants have a different response and the latter that their response is not significantly different.

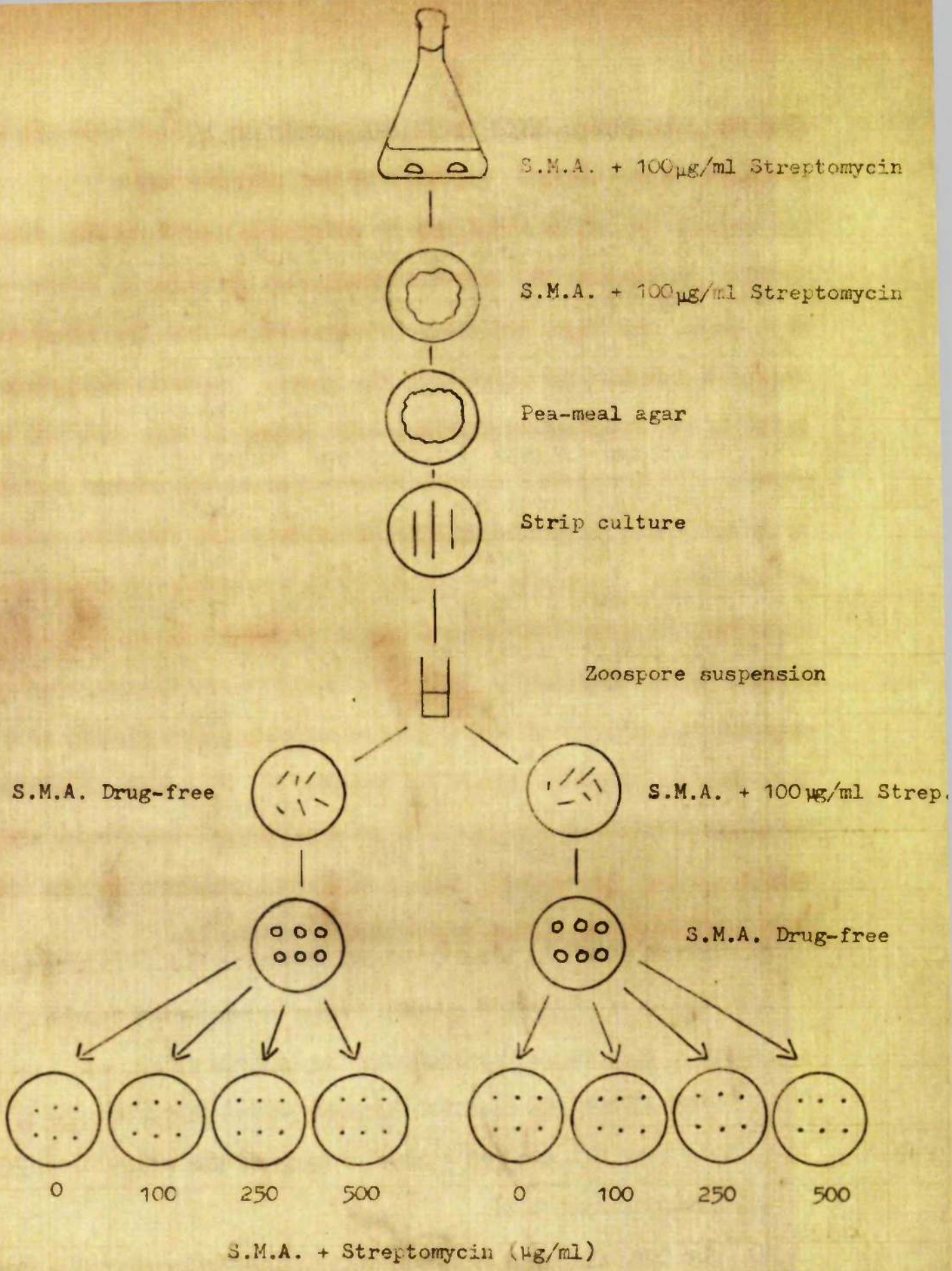


Figure 12. The method used to test tolerance of single zoospore isolates of streptomycin resistant strains.

Characterisation of Resistant Growths

Resistance to streptomycin.

Resistant growths were obtained using the liquid culture selection method. Four flasks of medium containing 100µg/ml streptomycin were each inoculated with approximately 10^5 zoospores and incubated for 10 days. Eight colonies appeared and were transferred to plates of S.M.A. containing 100µg/ml streptomycin. Plates of pea-meal agar were inoculated with each isolate to provide mycelium for strip cultures. Zoospores subsequently released from strip cultures were plated on S.M.A. and on S.M.A. + 100µg/ml streptomycin. Two isolates failed to sporulate satisfactorily and were discarded. Twelve germinated spores, of which six had been germinated on drugged medium and six on drug-free medium, were transferred to S.M.A. The resulting colonies provided inocula for testing on S.M.A. containing 0, 100, 250, and 500µg/ml of streptomycin (Figure 12).

The drug tolerance of the various strains was as follows:

1) Growth from all inocula on all concentrations of drug were comparable to wild-type growth. i.e. These twelve single zoospore isolates were uniformly resistant regardless of whether they were one of the six isolated from drug-free plates or one of the six from the drugged plates. Three of the original eight strains were like this.

2) The drug response of one strain was indistinguishable from wild-type. All twelve single zoospore isolates were uniformly sensitive.

3) The single zoospore isolates of one strain showed a range of tolerance. Some were resistant to 100µg/ml but sensitive to 500µg/ml while others were resistant to 500µg/ml. Variation in tolerance occurred independently of whether the isolates had been made from drugged or drug-free medium.

4) One of the eight strains failed to grow on pea-meal agar when hyphal inocula were transferred from the original resistant growth. This strain was streptomycin dependent as normal growth was obtained on the addition of 100µg/ml of streptomycin to the medium. Of the twelve single zoospore isolates of this strain, six of which had been rescued from drug-free medium before growth had ceased, all were found to be uniformly dependent.

The asexual progeny of the six strains which were examined all showed morphological variation. Some progenies were extremely variable in morphology, while others included a low frequency of variant colonies. The particularly distinct variation found in the streptomycin dependent strain will be dealt with in some detail in the next part of this thesis.

A single zoospore isolate of the streptomycin dependent strain and one from the least variable resistant strain, henceforth referred to as Sd and Sr, were selected for further study. Cultures were stored in one-ounce bottles of oatmeal agar, under paraffin oil, at room temperature. 100µg/ml streptomycin was included in the oatmeal agar for the Sd isolate.

Isolate		Replications	µg/ml of streptomycin in medium			
			0	100	500	1000
	Sr	1	47	53	64	65
		2	49	54	61	65
		3	56	63	63	68
	Sd	1	0	13	10	13
		2	0	7	7	8
		3	0	9	10	10
	Wt.	1	28	0	0	0
		2	30	0	0	0
		3	32	0	0	0

Table 14. Counts of colonies established from zoospores plated on media with different concentrations of streptomycin.

	Item	Df	Mean Square	F
Sr isolate	Between samples	3	137	9.785**
	Within samples	8	14	
Sd isolate	Between samples	2	1.3	
	Within samples	6	6.2	

Table 15. Analysis of Variance: Drug concentration and zoospore germination of Sr and Sd.

The drug response of asexual progeny of Sd and Sr isolates.

1) Colony establishment from zoospores.

Strip cultures of the Sd, Sr and wild-type isolates were made in the usual way except that 100µg/ml of streptomycin was included in the pea-meal agar for the Sd isolate. Zoospores suspended in 2% sucrose were counted and the concentration adjusted to approximately 3 x 10^2/ml. Three replicate plates at each streptomycin concentration were spread with 0.2 ml of suspension. The number of colonies counted after 72 hours incubation is recorded in Table 14.

Some care is needed in the interpretation of the results as zoospores had been suspended in 2% sucrose without physiological salts. It was later found (see page 22) that viability of zoospores decreases with increase in time under these circumstances. The plates spread first must have received fewer damaged zoospores. The increasing numbers of colonies of the Sd isolate on increasing concentrations of drug is probably due to the fact that zoospores were plated first into medium with 1000µg/ml streptomycin and then onto plates containing decreasing concentrations of drug. However, the results indicate that germination of Sr zoospores to form colonies is not inhibited by streptomycin at concentrations up to 1000µg/ml. Colonies are established from Sd zoospores at streptomycin concentrations between 100 and 1000µg/ml. On drug-free media Sd zoospores germinate and a limited branched mycelium is formed but when growth ceases the micro-colony quickly dies; it does not grow again when suitable amounts

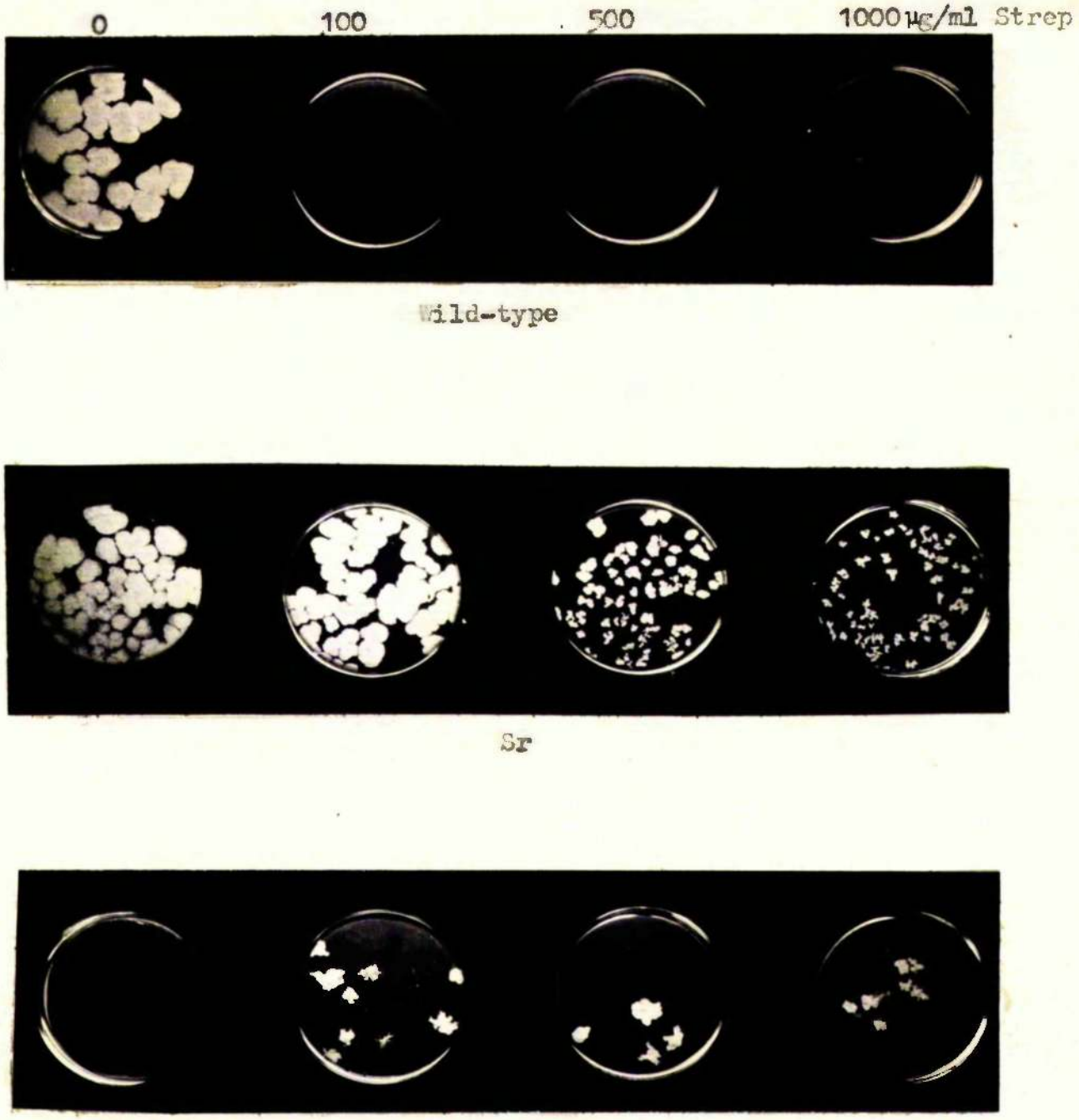


Figure 13. Zoospore platings on media with different concentrations of streptomycin.

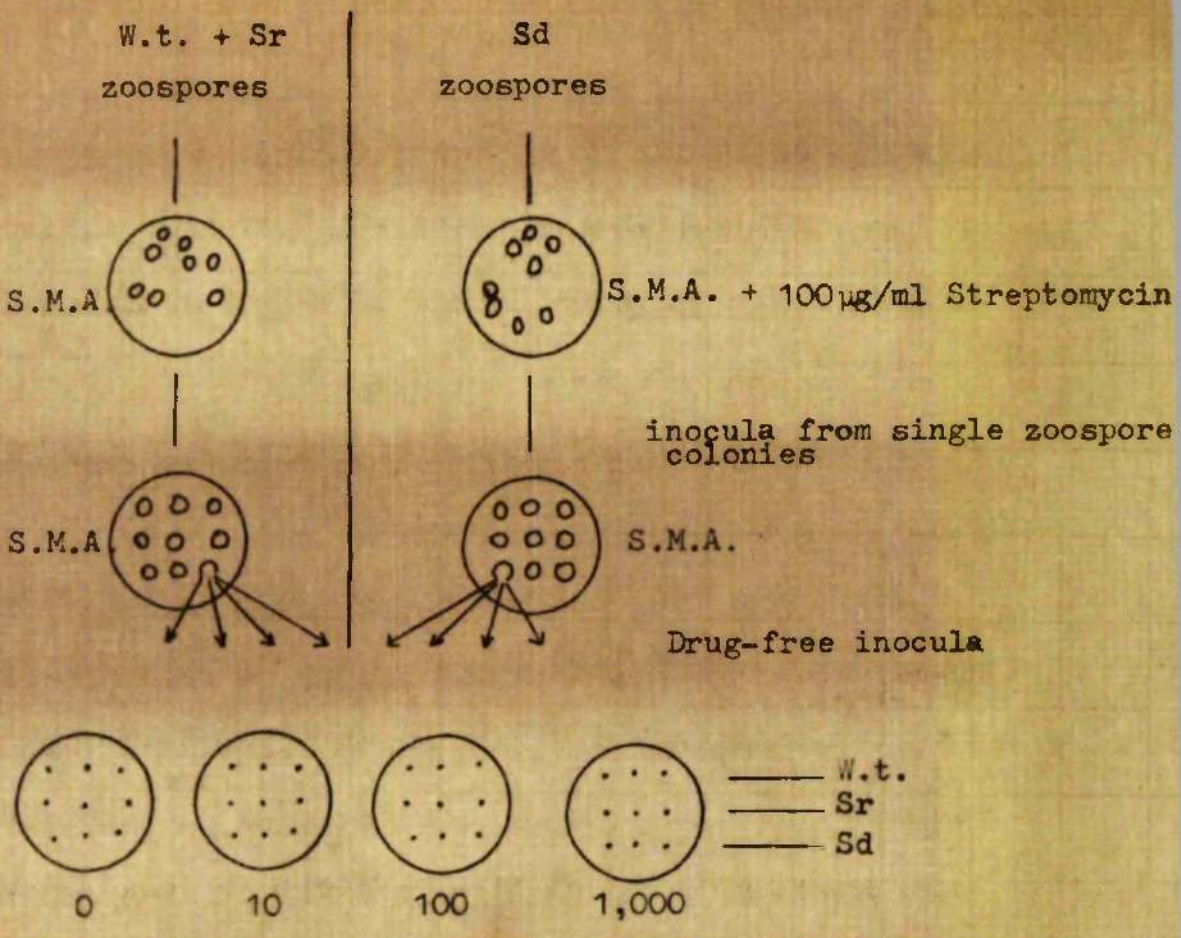


Figure 14. The method used to inoculate drugged plates with asexual progeny.

of streptomycin are added to the medium. Wild-type zoospores do not grow into colonies on media with 100µg/ml streptomycin or above. Zoospore colonies of Sr, Sd and wild-type on different concentrations of streptomycin are shown in Figure 13.

2) Dosage response of samples of the asexual progeny of Sd, Sr and wild-type isolates to streptomycin.

Wild-type and Sr zoospores were plated on S.M.A. and those of Sd on S.M.A. + 100µg/ml streptomycin. After incubation for 72 hours a 1 mm disc was cut from each of 50 colonies of each strain and transferred to plates of S.M.A. These cultures were grown on for 48 hours to provide drug-free inocula for testing on different drug concentrations. Sd inocula from drugged media make considerable growth on drug-free media until the streptomycin in the inoculum is exhausted. Four 1 mm discs were then transferred from each colony on drug-free medium to plates of S.M.A. containing 0, 10, 100 and 1000µg/ml streptomycin respectively. Each plate was inoculated with nine single zoospore isolates, three being zoospore isolates of Sr, three of Sd and three of wild-type, and was incubated 72 hours. The method of inoculating the plates is illustrated in Figure 14 and a typical set of plates is shown in Figure 15. The relative growth made by each inoculum was measured photometrically and the results are represented in Figures 16, 17 and 18.

Meter reading values (between 1 and 100) were transformed to logarithms and the numbers of individuals within equal but arbitrary

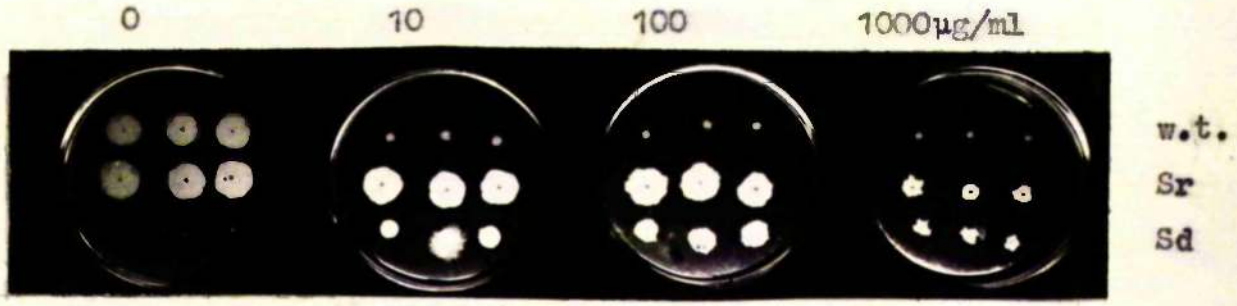


Figure 15. Response of asexual progeny to streptomycin.

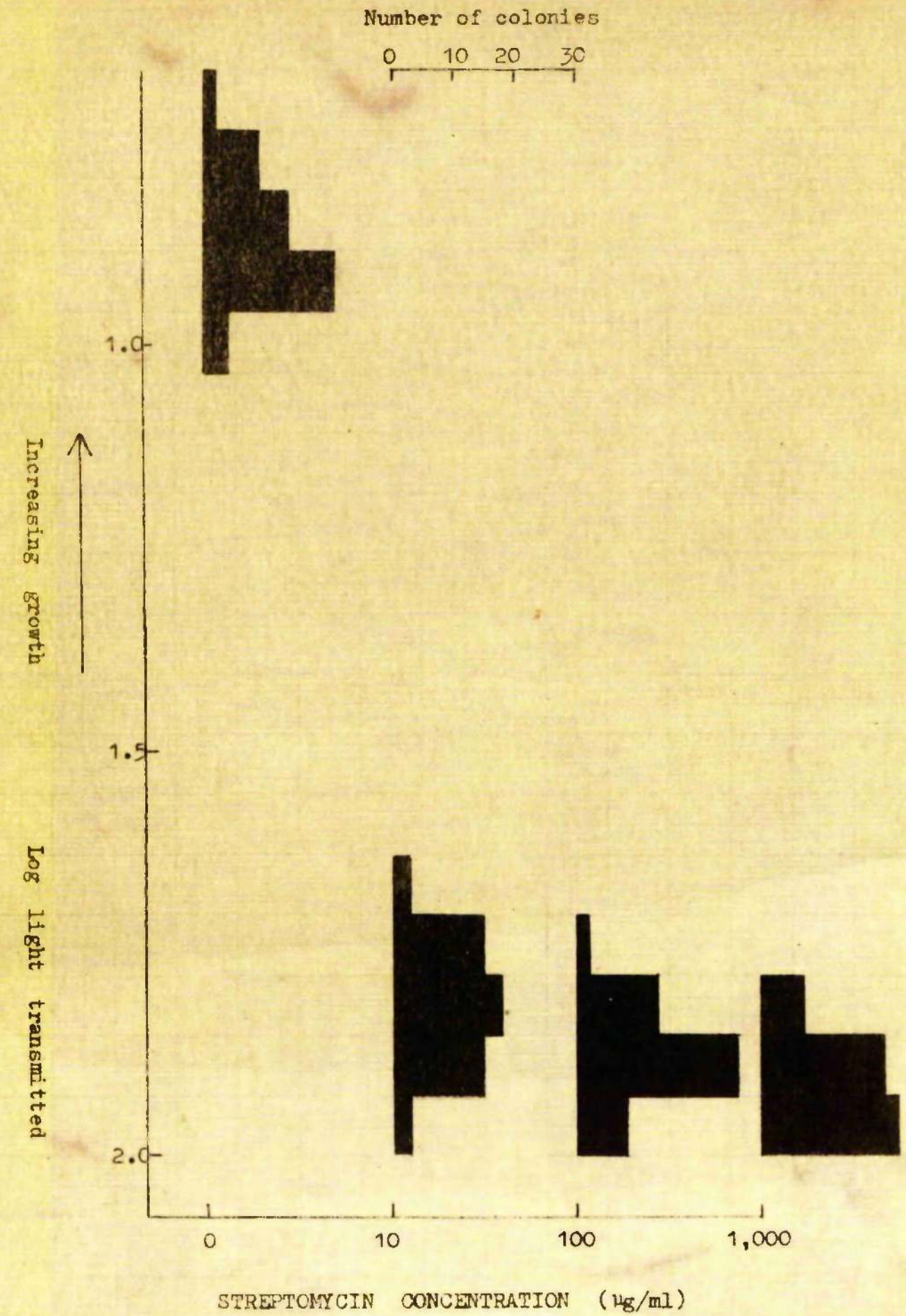


Figure 16. Response of asexual progeny of wild-type to Streptomycin.

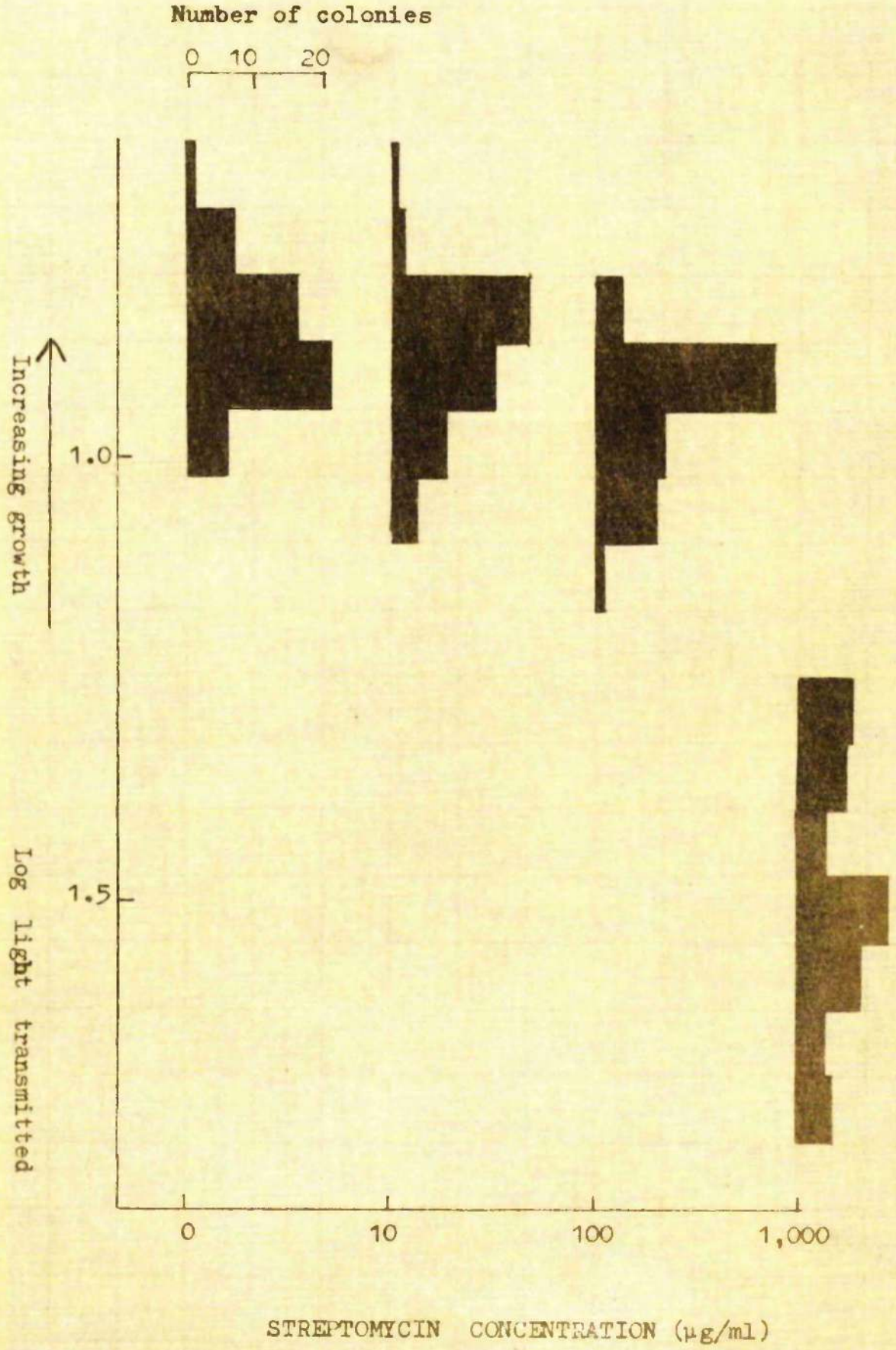


Figure 17. Response of asexual progeny of Sr to Streptomycin.

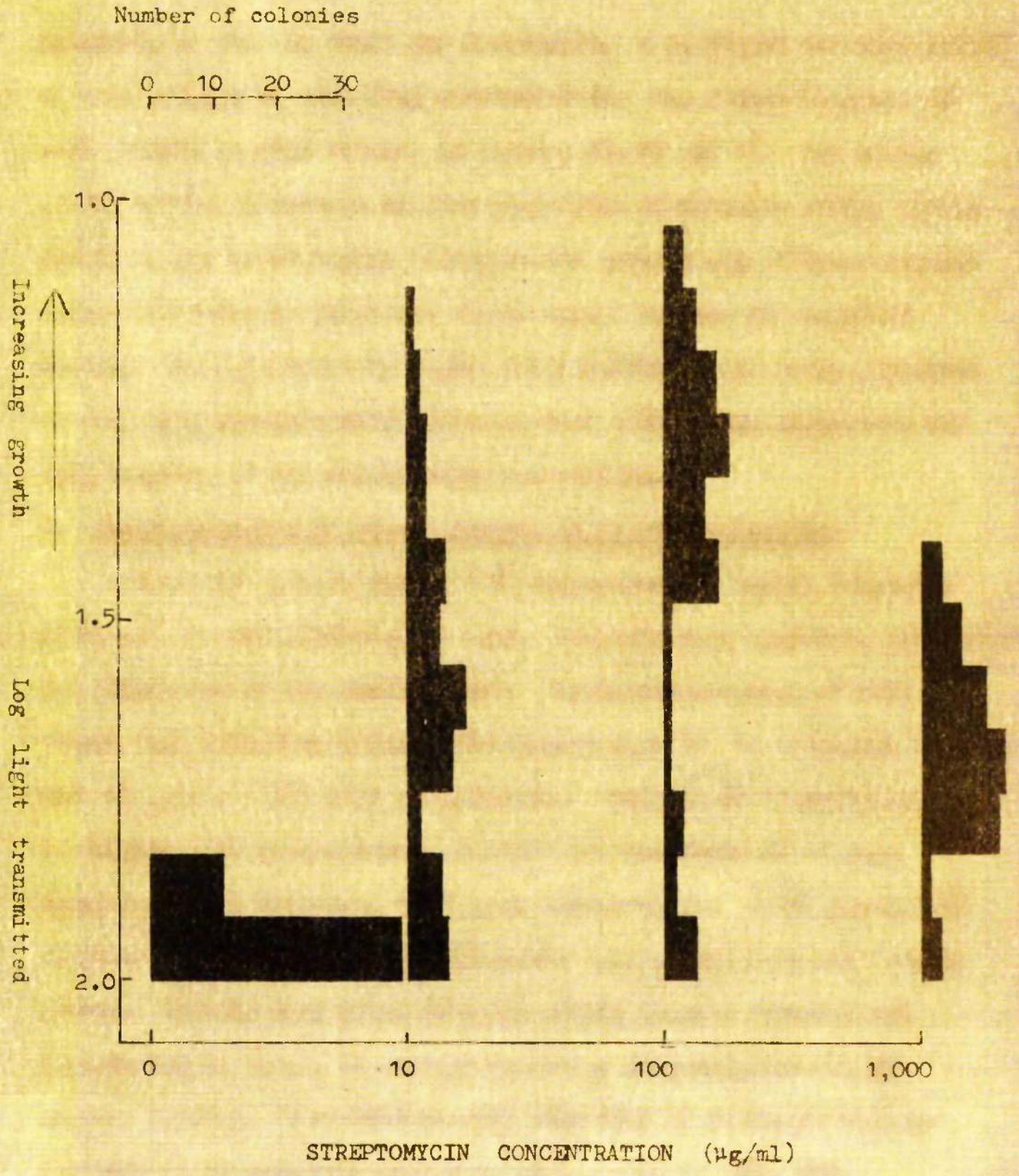


Figure 18. Response of asexual progeny of Sd to Streptomycin

intervals of the log scale was determined. A histogram was constructed of each progeny at each drug concentration. The dosage response of each progeny is also evident in Figures 16, 17 and 18. The slower growth of the Sd inocula and the proportion of colonies making little growth on any media merely indicates the greater lag of these inocula taken from colonies which had almost ceased to grow on drug-free medium. The approximately normal distribution of all three progenies at each drug concentration indicates that within each population the drug response of the sample taken was uniform.

3. Dosage response of sexual progeny of Sd and Sr isolates.

Oat-extract plates spread with suspensions of hyphal fragments of Sd and Sr were incubated 21 days. Oospores were digested, sterilized and germinated by the usual method. Germinated oospores of each strain were transferred from non-nutrient agar to 1) drug-free S.M.A., and 2) S.M.A. + 100µg/ml streptomycin. Oospores of Sr continued to develop into colonies on both media whereas those of Sd only formed colonies on S.M.A. + 100µg/ml streptomycin. Of 50 germinated oospores of Sd transferred to drug-free S.M.A. none made any further growth. Inocula were taken from all single oospore colonies and transferred to S.M.A. to provide uniformly drug-free inocula for further testing. 1 mm disc inocula from each of 50 single oospore isolates of Sd were transferred to plates with 0, 10, 100, and 1000µg/ml streptomycin respectively. Similarly inocula from each of 100 single oospore isolates of Sr were transferred to plates

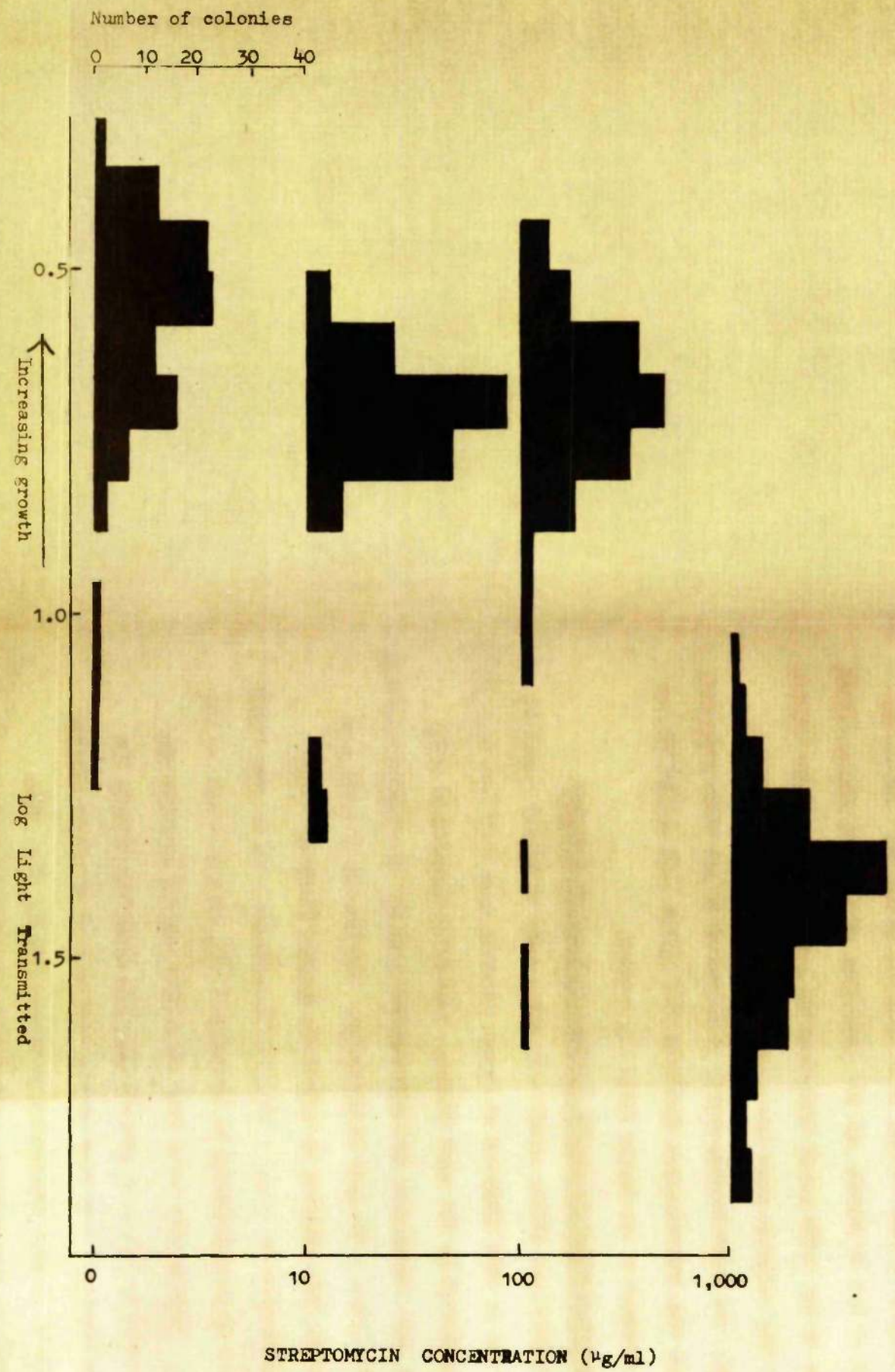


Figure 19. Response of sexual progeny of Sr to Streptomycin.

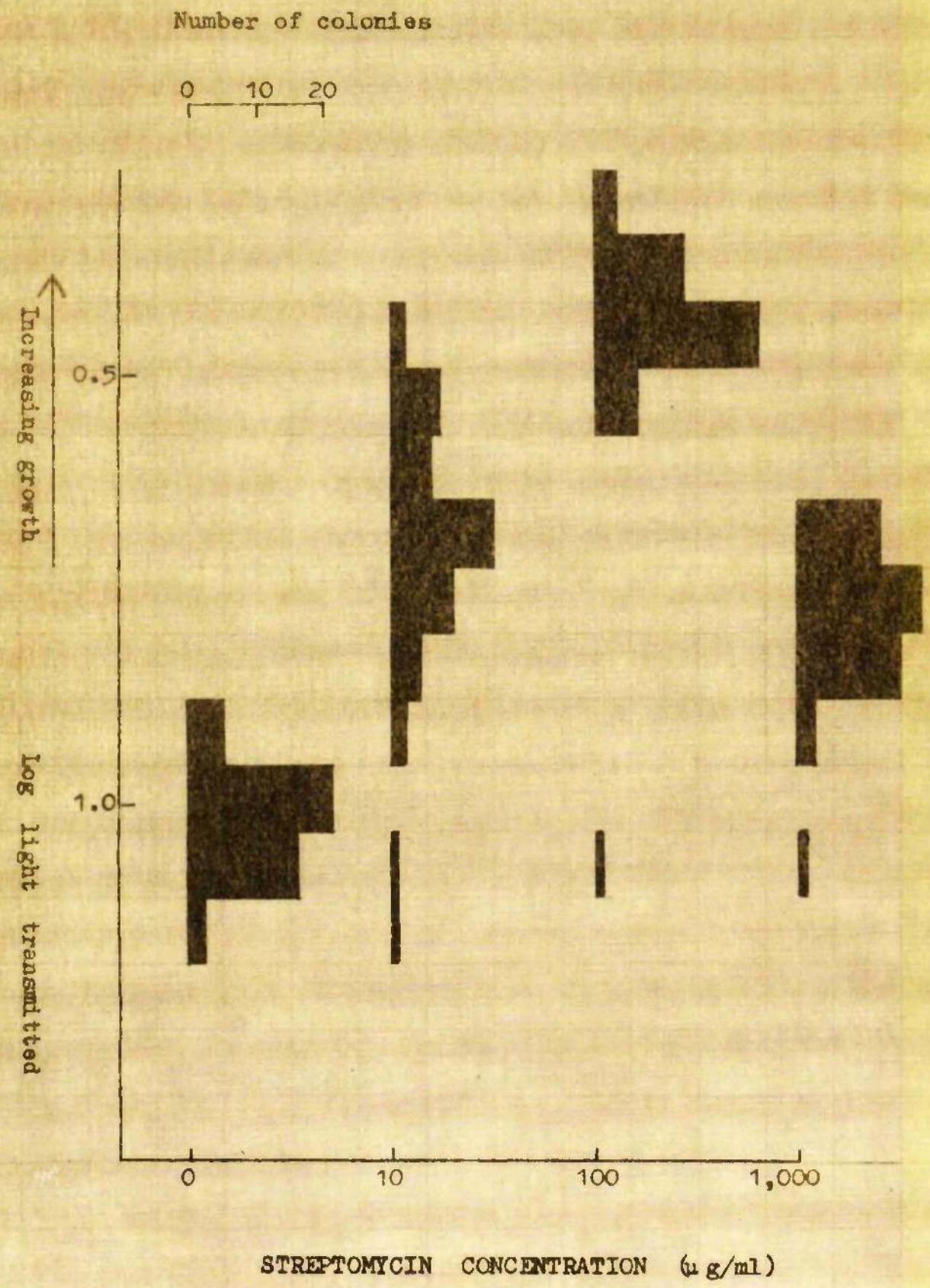


Figure 20. Response of sexual progeny of Sd to Streptomycin.

with 0, 10, 100, and 1000µg/ml streptomycin respectively. Relative absorption readings were taken after 72 hours incubation.

Results are represented graphically in Figures 19 and 20. Again histograms have been constructed to show the relative growth of the population of single oospore isolates at each drug concentration. The distributions which are approximately uni-modal at each concentration indicates that individuals of each strain are essentially similar in their response to streptomycin. There is no evidence of more than one response class in either of the sexual progenies. Comparing the dosage response of the sexual progeny of Sr and Sd with that of their asexual progeny (Figures 17 and 18) it is obvious that the response has been inherited in each case without change. It must be concluded that no segregation has occurred during sexual reproduction in either strain.

An attempt is being made to cross the Sr and Sd strains. Oat-extract plates have been inoculated with 1) disc inocula, 2) mixed hyphal fragments of each strain. If hybrid oospores are formed these might give rise to segregant and recombinant progeny with an altered drug response. Progeny from the cross have not yet been sampled.

<u>The apparent inhibition of resistant individuals by a population of sensitive organisms</u>.

The phenomen of self-inhibition of spore germination is well-known in work with <u>Puccinia</u> spp. (Yarwood 1956 and others), and has been reported in studies on the germination of bacterial endospores (Stedman

et al. 1956). Grigg (1952) reported that prototrophic spores of Neurospora were inhibited when plated on minimal medium with large numbers of auxotrophic spores. Working with penicillin resistance in pneumococci and streptococci, Gaz and Eagle (1953) were able to demonstrate that the numbers of resistant colonies appearing in platings of sensitive cells on drugged medium decreased as the numbers of cells plated increased. Thus resistant colonies were found in plates of 10^6 to 10^8 cells, but none were found in plates containing 10^9 cells. When resistant cells were added to platings of sensitive cells the numbers recovered decreased in plates with increasing numbers of sensitive cells, from 10^6 to 10^8, while no resistant colonies appeared even when 2,000 resistant cells were added to 10^9 sensitive cells. The effect was only apparent on solid medium. Sager (1962) reported that chromosomal gene mutants resistant to streptomycin in Chlamydomonas were suppressed 10-fold when the number of cells rose to 5 to 10 x 10^6 per plate. The appearance of non-chromosomal mutants was not suppressed at 10^7 cells per plate or less.

An experiment was designed to determine if a similar inhibition could prevent the growth of resistant mutants in a sensitive population of P. cactorum. A small number of Sr zoospores were added to drugged media containing large numbers of wild-type zoospores.

1) On solid medium. Suitable dilutions of a suspension of sensitive wild-type zoospores were plated on S.M.A. + 100µg/ml streptomycin to give plates containing approximately 10,000,

1,000, 100 and 10 spores. Approximately 30 zoospores (0.1 ml of a suspension) was added to each plate and to control plates without wild-type spores. Six replicate plates were spread at each spore concentration.

2) <u>In liquid medium</u>. Six 100 ml flasks containing 50 ml of standard liquid medium + 100µg/ml streptomycin were inoculated with 50,000, 5,000, 500 and 50 sensitive wild-type zoospores respectively. Exactly 0.1 ml of Sr zoospore suspension (approximately 30 spores) was added to each flask and to control flasks without wild-type spores. Six replicates were prepared of each treatment.

Counts were made of resistant colonies after 72 hours incubation and results are recorded in Table 16.

		Number of wild-type spores present per culture									
		Liquid Medium					Solid Medium				
		0	50	500	5,000	50,000	0	10	100	1,000	10,000
Replicates	1	0	1	2	1	2	0	4	2	2	2
	2	4	4	2	5	0	8	8	3	3	1
	3	1	4	3	6	3	3	10	3	1	1
	4	5	4	3	4	3	4	3	9	2	3
	5	4	8	7	3	0	8	3	5	2	2
	6	9	5	6	3	3	6	8	6	5	2
Totals		23	26	23	22	11	39	36	28	15	11

Table 16. Colonies established from surviving Sr zoospores in populations of inhibited wild-type spores.

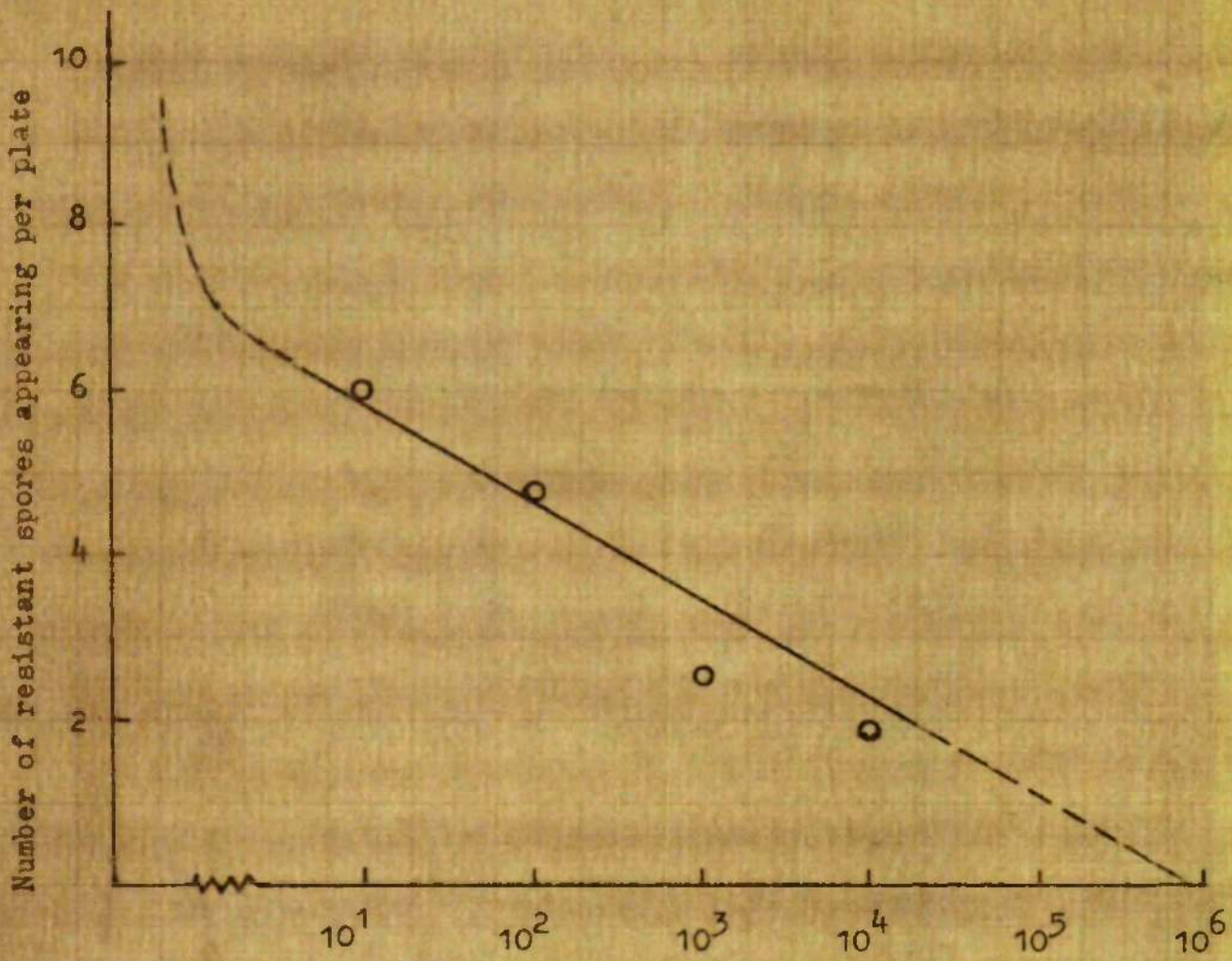


Figure 21. The relationship between the size of the wild-type population and the numbers of resistant colonies established.

Item	Df	Mean Square
Between samples	4	2.865
1) Between 0 & rest	1	7.30
2) Within rest	3	1.332
Regression	1	4.05
Remainder	2	0.055
Within samples	25	0.098

Table 18. Analysis of Variance - solid medium.

	Item	Df	Mean Square	F
Solid Medium	Between samples	4	2.86	12.13*
	Within samples	25	0.236	
Liquid Medium	Between samples	4	0.415	1.04
	Within samples	25	0.40	
	Between samples			
	1) Between 30,000 & rest	1	1.63	4.07
	2) Within rest	3	0.01	
	Within samples	25	0.40	

Table 17. Analysis of Variance: on data transformed to $(\frac{1}{2} + y)^{\frac{1}{2}}$

The numbers of colonies, y, per replicate was transformed to $(\frac{1}{2} + y)^{\frac{1}{2}}$. An analysis of variance of the data (Table 17) indicates that there is a significant difference between treatments on solid medium. The mean number of resistant colonies per plate was plotted against the logarithm of the number of wild-type spores per plate (x) and the regression line fitting the points from $x = 10$ to $x = 10^4$ was calculated (Figure 21). The analysis of variance (Table 18) indicates that the relationship is linear over this range. From the calculated regression line the value of x when $y = 0$, i.e. when no resistant spores would appear, was estimated to be $10^{5.8}$. However it is unlikely that the relationship would continue as a straight line with this slope.

The analysis of variance (Table 17) indicates that there is a

significant suppressive effect in liquid medium only when the number of wild-type spores present was 50,000 per culture but not when the number was 5,000 or less. It is interesting to note that Saz and Bagle (1952) only found a suppressive effect in solid medium. To reduce possible suppression in liquid medium, spores could be kept in suspension by using a magnetic stirrer to circulate drugged liquid medium in flasks.

The stability of streptomycin dependence in the Sd strain.

The original isolates were stored in one-ounce bottles of oatmeal agar + 100µg/ml streptomycin under paraffin oil. Inocula from these storage cultures were always found to be uniformly dependent and have been so for some 18 months. To determine if "mutation" to non-dependence occurs Sd was cultured in streptomycin-free media.

1) Selection of non-dependent growths on S.M.A. Six plates of S.M.A. were each inoculated centrally with a 4 mm disc cut from a colony of Sd growing on S.M.A. + 100µg/ml streptomycin. Initial growth compared favourably with that made by similar discs placed on S.M.A. + 100µg/ml streptomycin, but as the streptomycin in the inoculum became exhausted growth slowed down and eventually stopped when the colonies were approximately 40 mm in diameter. No further growth took place until it was observed that growing sectors were arising from the colony edge in all six plates. These non-dependent sectors arose some 20 days after the initial inoculation. Figure 22 shows a non-dependent

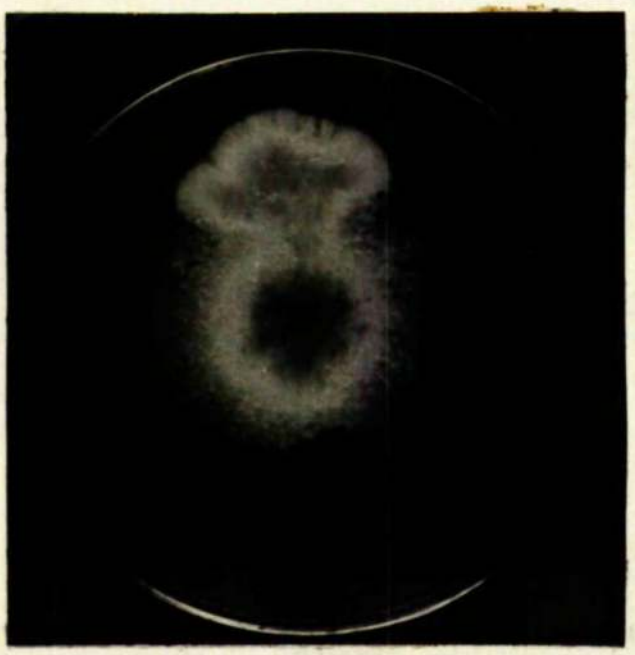


Figure 22. A streptomycin independent sector arising from a non-growing dependent colony on drug-free medium.

sector arising from a dependent colony which had ceased to grow.

2) Selection of streptomycin independent colonies derived from zoospores of the Sd strain. Four 2 litre flasks were prepared with 500 ml of standard liquid medium. 5 ml volumes of zoospore suspension of the Sd strain, prepared from strip cultures of mycelium grown on pea-meal agar + 100µg/ml streptomycin, were added to each flask. The number of zoospores placed in each flask is recorded in Table 19. Flasks were incubated for five days when counts were made of non-dependent colonies which appeared (Table 19). The frequency of non-dependent colonies arising

	Flasks			
	1	2	3	4
Number of zoospores added	8×10^5	1.7×10^6	8×10^5	8×10^5
Number of growths appearing	2	4	3	4

Table 19. Numbers of non-dependent colonies arising in flasks of undrugged medium.

under these conditions was between 2×10^{-5} and 4×10^{-5} per zoospore. All non-dependent colonies tolerated streptomycin concentrations up to 1000µg/ml i.e. the change was from dependence to resistance.

The Development of Resistance to other Drugs.

As the reconstruction experiment described above indicated that the appearance of resistant colonies may be suppressed in platings of sensitive zoospores, selection by the plate spreading method was discontinued. In a series of selection experiments attempts were made to isolate mutants resistant to various inhibitors using the liquid culture and sandwich plate methods. Colonies found growing in selective medium which inhibited the formation of wild-type colonies were isolated and tested for increased tolerance and persistence of any resistance. Firstly, isolations were made onto agar plates containing the same concentration of drug used to select the growths and if resistance were still apparent, inocula were transferred to drug-free S.M.A. and to pea-meal agar. After three days growth on S.M.A. the drug response was compared with wild-type and with the isolate which had been kept growing on drugged agar. The drug response of zoospores produced from strip cultures of mycelium grown on pea-meal agar was compared with that of wild-type zoospores. Zoospores were irradiated with ultraviolet light to give 99.9% kill and were used in some liquid culture selection experiments.

Selection by the liquid culture method.

Experiment 1. Two flasks with selective doses of each of the following inhibitors were prepared: acriflavin, malachite green,

p-fluorophenylalanine, sulphanilamide and tetracyclin. Zoospore inoculations were made as previously described and flasks were incubated for six days. One growth was isolated from a flask with malachite green and two from a flask with sulphanilamide. No growths appeared in other flasks. None of the isolates made further growth on transfer to S.M.A. with the same selective doses of inhibitor. Their drug response was found to be indistinguishable from wild-type.

Experiment 2. In a series of selections using acti-dione, p-fluorophenylalanine and sulphanilamide, the medium in certain flasks was circulated slowly with a magnetic stirrer to prevent zoospores settling in a layer on the bottom of the flasks and possibly suppressing the growth of resistant zoospores. Also certain flasks were inoculated with UV-irradiated zoospores (99.9% kill). Six flasks containing 500 ml of medium were used for each drug. Two flasks of drugged medium were each inoculated with approximately 10^5 unirradiated zoospores. Two flasks of drugged medium and two of drug-free medium were each inoculated with approximately 10^5 irradiated zoospores. One of each pair of flasks receiving a different treatment was stirred during incubation. After incubation for six days any resistant growths appearing were isolated on drugged S.M.A.

p-fluorophenylalanine. No resistant growths appeared in any flasks.

sulphanilamide. Growth appeared in all flasks containing drugged medium (Table 20).

	Drugged Medium		Drug-free Medium
	No UV	UV	No UV
Unstirred	23	3	many
Stirred	5	2	many

Table 20. Numbers of growths appearing in flasks with and without sulphanilamide.

Ten colonies from the non-irradiated unstirred flask and the ten from the other three flasks were isolated. Sixteen of the twenty isolates made no further growth on selective S.M.A. but four seemed to have adapted allowing them to grow slowly. The latter were sub-cultured on drug-free S.M.A. and transferred back to selective S.M.A. on which their response was no better than that of wild-type. Zoospores of each of the four isolates and of wild-type were plated on different concentrations of S.M.A. + sulphanilamide but no increased tolerance was observed.

acti-dione. Approximately twenty growths were observed in the stirred culture which had been inoculated with non-irradiated zoospores.

No growths appeared in other flasks of drugged medium. Each of the twenty growths was isolated on S.M.A. containing the same concentrations of drug as the liquid selection medium but in all cases further growth was inhibited. On testing the drug response of inocula from inhibited growths retained on the selective S.M.A. and from sub-cultures of these on drug-free S.M.A. it was found that all inocula from the growths on drugged media made more growth on media with sub-inhibitory levels of drug than inocula from the sub-cultures on drug-free medium. In fact the response of the latter was similar to wild-type.

It was thought that determinants conferring resistance may have been selected against during sub-culture on drug-free medium. Accordingly growths with increased tolerance were grown on pea-meal agar containing a concentration of acti-dione inhibitory to wild-type but tolerated by resistant growths. The zoospores from these cultures germinated and formed colonies on media containing levels of drug which inhibited wild-type zoospores or those of resistant growths cultured on drug-free pea-meal agar. However isolates from these apparently resistant single zoospore colonies had a wild-type drug response after sub-culture on drug-free medium. It was concluded that the resistance of zoospores was temporary and could be easily lost by growth in undrugged medium.

Selection by the sandwich plate method.

S.M.A. containing selective concentrations of acti-dione and patulin were used to overlay plates of germinated zoospores as described

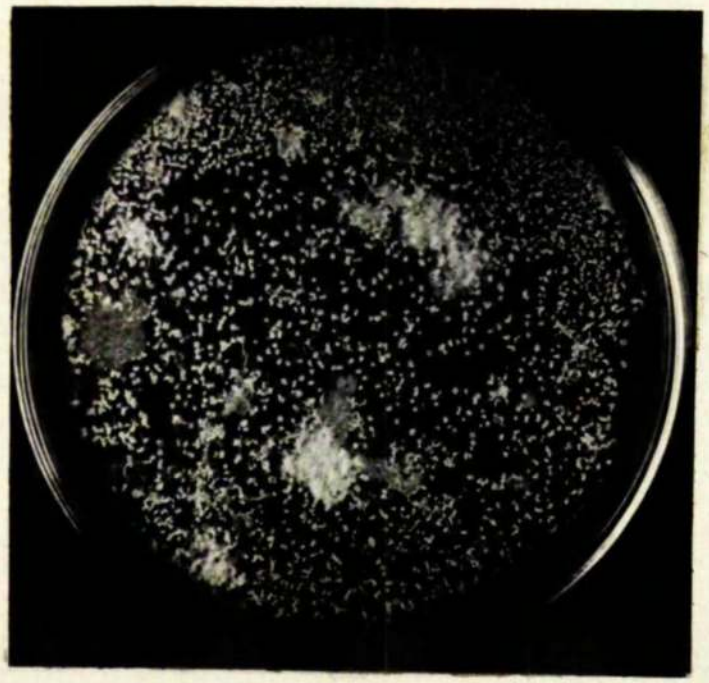


Figure 22a. Resistant growths on sandwich plate of acti-dione.

previously. Seventy plates of germinated spores were used in each selection attempt. Thirty-five cultures were overlain with drugged agar directly while the remaining half of the cultures were irradiated with ultraviolet light before overlay. It was found that irradiation for 40 seconds was suitable, allowing the appearance of 30 to 40 colonies on the agar surface four days after overlay with drug-free medium.

Experiment 1. In the first selection experiment the drugged agar was added to cultures immediately after irradiation.

acti-dione. Many hundreds of small knots of hyphae were formed at the interface of the two layers of agar in each plate after 48 hours incubation. Four days later it was found that certain of these knots had given rise to a mycelium which had penetrated to the surface of the agar. The ten to twenty colonies which thus developed on the surface of each plate had variable morphologies. (See Figure 22). In irradiated plates hyphal knots and surface growths derived from these were also observed but were much less frequent. Inocula from a sample of surface growths were tested as previously described for resistant growths isolated from liquid medium. Again the increased tolerance did not persist in sub-cultures on drug-free media.

patulin. Irradiated and non-irradiated cultures were overlain with S.M.A. containing selective concentrations of patulin.

Cultures were incubated seven days before any growths appeared on the surface. Five non-irradiated cultures were each then found to have a single growth arising from the edge of the plate. Inocula from these growths responded as wild-type inocula on plates of S.M.A. containing a range of concentrations of patulin.

Experiment 2. In the second set of selection experiments half the cultures were irradiated for 40 seconds after the usual 24 hours incubation. All plates were then incubated a further eight hours at 24°C before drugged agar medium was added.

acti-dione. Adaptive growths appeared on the agar surface with approximately the same frequency as in Experiment 1. The increased tolerance of a sample of isolates from irradiated cultures was again found to be temporary, disappearing after culture on drug-free medium.

patulin. No growth had appeared on the surface of any plate after incubation for ten days.

Discussion

The inactivation of zoospores by ultraviolet irradiation.

In some organisms it is possible to identify haploid and diploid strains by the shape of the killing curve. For example, an exponential, single hit curve is obtained with haploid yeast whereas similar diploid strains give a sigmoidal, multiple hit curve indicating that more than one target needs to be inactivated before the cell is killed (DeLong & Lindegren 1951). Norman (1951) showed that the number of nuclei in Neurospora conidia could be estimated with some accuracy by extrapolating the linear part of the killing curves for each spore sample to meet the survival axis. However these examples assume that nuclear inactivation is responsible for the lethal action of UV. It is likely that in other cases extra-nuclear damage contributes to inactivation. Witkin (1947) showed that a single mutation could alter sensitivity and the form of the killing curve in UV studies with E. coli. It is therefore not always possible to correlate estimates of target number with numbers of nuclei or with the ploidy of single nuclei.

Extrapolation of the curve obtained here (Figure 9) indicates an inactivation of many hundreds of targets. This may reflect the criterion used to estimate survival or may indicate extra-nuclear inactivation. Extrapolation of Buddenhagen's curve for survival

in P. cactorum (Buddenhagen 1958) gives an estimate of four for the number of targets but as was pointed out previously (page 64) the slope of his curve may have been influenced by poor zoospore survival in water and by suppression in high density platings. It may be concluded that it is not possible to predict the ploidy of the nuclei with the evidence available.

The origin of the increased tolerance to drugs found in P. cactorum.

In the search for drug resistant mutants two types of increased tolerance have been observed. i.e. the stable heritable changes to streptomycin resistance and dependence and the temporary increase in tolerance to acti-dione and sulphanilamide. Lacking further evidence it is only possible to speculate on possible modes of origin of these changes and to erect hypotheses around the observed facts.

Streptomycin resistance and dependence. The observed facts are these:

1) No segregation was observed in the asexual or sexual progeny of resistant or dependent strains even although morphological segregants appeared in both the asexual and sexual progenies of the dependent strains.

2) The frequency of mutation per zoospore from streptomycin sensitivity to any specific level of streptomycin resistance has not been measured accurately but in one selection experiment

(page 60) involving not more than 5×10^5 zoospores several resistant strains were recovered. The frequency of non-dependent zoospores in a population of streptomycin dependent zoospores was shown to be between 4×10^{-5} and 2×10^{-5} per spore. This frequency may not be a valid estimate of reverse mutation from dependence as mutation of a multiplying determinant occurring during growth of the mycelium could have given rise to several non-dependent spores.

The above evidence could be taken to support an interpretation based in either (a) nuclear or (b) cytoplasmic control of resistance and dependence.

(a) Resistance and dependence arose by mutation of chromosomal genes. The absence of segregants in the sexual progeny of both strains would indicate that the somatic nuclei are haploid. Diploid nuclei would need to be homozygous otherwise segregation would have occurred; it is unlikely that rare homozygous mutants were selected. The possibility that oospores were formed parthenogenetically has not been excluded.

Estimated frequencies of spontaneous reverse mutations for various inositolless loci in Neurospora crassa range from 1.4×10^{-4} to 1.4×10^{-10} per microconidium (Giles 1951), and gene mutation to streptomycin resistance in Chlamydomonas occurred with a frequency of between 8×10^{-5} to 1.1×10^{-6}

per cell per division (Sager 1962). The frequency of mutation varies from organism to organism and from gene to gene and so if the estimate of spontaneous mutation to non-dependence in P. cactorum is valid then it could be taken to reflect the frequency of mutation of a nuclear gene. The change to resistance from sensitivity may have been induced by streptomycin but there is no evidence in the literature to suggest that streptomycin can act as a mutagen for chromosomal genes.

(b) Streptomycin resistant and dependent strains are non-chromosomal mutants. There is no reason to suspect that a phenotype controlled by cytoplasmic determinants cannot be inherited by all the progeny during selfing. The inheritance of extra-chromosomal differences has been studied mainly in bi-sexual or heterothallic organisms. Outcrosses with wild-type have shown maternal or uniparental inheritance e.g. streptomycin resistance and dependence in Chlamydomonas (Sager & Yoshihiro 1961), a hundred per cent transmission of the mutant character e.g. suppressive petite yeast (Ephrussi et al. 1955) or complete absence of the mutant phenotype in the sexual progeny e.g. neutral petite yeast (Ephrussi 1953). If self-replicating cytoplasmic determinants are involved the mutant phenotype may have arisen by loss or change of determinants. Absence of wild-type segregants would indicate that wild-type determinants were no longer present in the cytoplasm. Rates of origin of cytoplasmic mutants are extremely variable. Petite

variants may be induced in all yeast cells treated with acriflavin (Ephrussi 1953) and Pittman (1957) has shown that 25% of yeast cells surviving an ultraviolet treatment were non-chromosomal petites. However frequencies are not always as high. The frequency of non-chromosomal mutation to streptomycin resistance in Chlamydomonas was shown to be affected by nutritional factors but varied between 3.7×10^{-9} to 3.4×10^{-7} per cell per division (Sager 1962). The estimated mutation rate observed in P. cactorum then, is consistent with an explanation based on mutation of cytoplasmic determinants. It is not possible to predict whether the changes observed, if cytoplasmic, were spontaneous and merely selected or whether they were induced by streptomycin. It has been shown that streptomycin can act as a non-specific inducer of various cytoplasmic mutations in Chlamydomonas (Sager 1962) and some evidence has been presented which indicates that streptomycin can induce unstable morphological changes in P. cactorum (see page 99). Since morphological differences, thought to be cytoplasmic, segregate independently of streptomycin dependence (page 110) the two sets of cytoplasmic determinants would require to be unlinked i.e. sited on different self-reproducing particles.

Studies on the action of streptomycin have suggested that resistant and dependent bacteria have ribosomes with a changed structure which alters the affinity of these organelles for the streptomycin molecule (Spotts & Stanier 1961). It is possible that, as seems to be the case

with Chlamydomonas, either the nuclear or the cytoplasmic system could control the inheritance of ribosomal differences. Without further evidence it is impossible to say which of the above hypotheses is the more likely to explain the situation in P. cactorum.

Resistance to acti-dione and sulphanilamide

The increased tolerance to acti-dione and sulphanilamide could hardly be attributed to a change in heritable material either in the nucleus or in the cytoplasm. When the drugs are removed the phenotype rapidly becomes identical to wild-type. This behaviour could result from the selection or induction of a cytoplasmic state allowing growth to continue on medium which prevents the growth of wild-type. The phenomenon is in some respects similar to the adaptations described by Dean and Hinshelwood (1954) and others, which allow the growth of Aerobacter aerogenes to grow in media with formerly toxic concentrations of proflavine. It also resembles induced enzyme synthesis which ceases when the inducer is removed (Rickenberg et al. 1953).

The failure to select other drug resistant mutants

There are several possible reasons why stable changes in tolerance were rarely observed.

1) Too few nuclei may have been screened for drug resistance. Mutation to resistance may occur with a frequency much lower

than 1 x 10^{-6} per zoospore. If nuclei were diploid and only dominant mutations to resistance expressed, the chance of finding one might be very small. The screening tests therefore may not have been carried out in a large enough scale to permit selection of rare mutations. The sandwich plate technique which allowed the screening of larger numbers of nuclei was no more successful. It is possible that if a mutation conferring resistance occurred in one nucleus of a germinated zoospore, the other wild-type nuclei in the heterokaryon so formed could prevent expression of the resistant phenotype.

2) P. cactorum. may have a high sensitivity to UV irradiation. There was no evidence to suggest that ultraviolet treatments had induced mutation to drug resistance but high frequencies of morphological changes, thought to be cytoplasmic in nature occurred among the survivors. It is possible that the cytoplasm is very sensitive to ultraviolet light and that doses of radiation below that required to induce nuclear gene mutation might cause lethal damage in the cytoplasm. Strains resistant and sensitive to UV irradiation are known in E. coli (Witkin 1947).

3) Drugs may prevent the initiation of mutations following UV treatment. It has been shown that the genetic consequences of UV irradiation can be modified during a sensitive period prior to the first post-irradiation cell division in bacteria and actinomycetes (Novick & Szilard 1949; Newcombe 1955; Berrie 1953). Induced mutations may be completely suppressed or their

usual frequency greatly enhanced by varying environmental conditions during this "sensitive period". RNA and protein synthesis are necessary for the initiation of DNA replication which precedes the appearance of mutations after UV treatment (Haas & Boudney 1959). Thus inhibition of protein synthesis by chloramphenicol during the "sensitive period" in E. coli suppressed the induction of back mutation to prototrophy (Witkin 1959). It is interesting to note that mutations from sensitivity to streptomycin resistance in E. coli display no "sensitive period" (Witkin & Theil 1960).

The antibiotic effect of acti-dione has been shown to be due to inhibition of DNA synthesis (Kerridge 1958). Sulphanilamide is thought to interfere with DNA synthesis by inhibiting folic acid and purine metabolism (Prusoff et al. 1948). The anti-metabolite p-fluorophenylalanine competes with its analogue phenylalanine during protein synthesis (Mitchell & Niemann 1947).

4) The expression of mutations conferring resistance may have been suppressed. As already pointed out, the appearance of certain mutants which are rarely selected can be increased by altering the physical and chemical composition of the medium to favour their expression. Evidence has been obtained that resistant spores may be suppressed in the presence of a sensitive population.

Methods used to select drug resistant mutants could perhaps be improved by modification along the following lines.

a) Larger numbers of nuclei could be screened, either by the use of massive strip cultures for producing large volumes of zoospore suspension, or more easily by using suspensions of hyphal fragments which are readily produced by blending freshly grown mycelium in "sucrose-salt" solution. Large volumes of suspension containing up to 10^5 viable fragments per ml can be easily obtained.

b) Precautions must be taken to prevent suppression of mutants. Large volumes of liquid medium should be used and stirring may help to keep spores or hyphal fragments in suspension.

c) Chemical mutagens which may not produce a "sensitive period" after treatment and which may not induce high frequencies of morphological variants could be used. In preliminary experiments ethylmethanesulphonate did not induce morphological changes in surviving zoospore colonies. Nitrous acid and mutagenic base analogues might prove to be useful.

d) When UV irradiation is used, time should be allowed for DNA to replicate before inhibitors are added to cultures.

SECTION III

MORPHOLOGICAL VARIATION IN *P. CACTORUM*

INTRODUCTION

Heritable changes which affect the morphology of fungal colonies growing on agar are frequently noted and the variant phenotypes can be isolated without using elaborate selection techniques. Morphological variation affects colony size, density and shape, the amount of aerial mycelium and the size, frequency and shape of reproductive structures. Macroscopic characters often reflect differences in growth rate or in the size, shape and frequency of branching of the hyphae. Variants may appear as sectors in colonies during vegetative growth or they may appear in asexual or sexual progeny.

Numerous reports can be found in the literature describing the segregation of morphological variants from wild isolates but in many cases the genetic basis of the variation remains uncertain. Crossing experiments have indicated that the variation may have a nuclear basis e.g. the colonial variant of Neurospora used in plating techniques differs from the wild-type at a single gene locus (Barratt & Garnjobst 1949). Mitchell et al (1953) studying the inheritance of respiratory deficiencies in Neurospora which resulted in abnormal morphologies showed that in certain cases the mutant phenotype showed a 1 : 1 segregation in reciprocal crosses with wild-type whereas in others (the mi and poky strains) the deficiency followed strict maternal inheritance. Similarly the petite phenotype in yeast may show nuclear or cytoplasmic inheritance (Sherman & Ephrussi 1962 and others).

Even when the mode of inheritance has been established as cytoplasmic the extent of variation and its expression are dependent on the genotype of the organism. Interactions of this kind are known where a nuclear gene can influence the expression of a character normally inherited extra-chromosomally e.g. the nuclear suppressor of poky in Neurospora (Mitchell & Mitchell 1956).

Thus most of the observed sectoring and somatic segregation in fungi may be due to:

1) The segregation of homokaryons from heterokaryons or of homozygotes derived from heterozygous diploid nuclei.

2) The segregation of cytoplasmic differences from a mixed cytoplasm.

Jinks (1959) has shown that the so called "dual phenomenon" in imperfect fungi may be based on either system. Of four species of Penicillium he examined, differences between segregants was shown to be nuclear in two species but cytoplasmic in the others.

Morphological Variation in the Peronosporales.

Several references to changes in morphological characters in Phytophthora spp. have appeared in the literature. Leonian (1925) in a fairly extensive study of variation and its inheritance in Phytophthora parasitica rhei described how a single sporangium culture sectored spontaneously giving five distinct morphological variants three of which remained constant during further transfer by single sporangia.

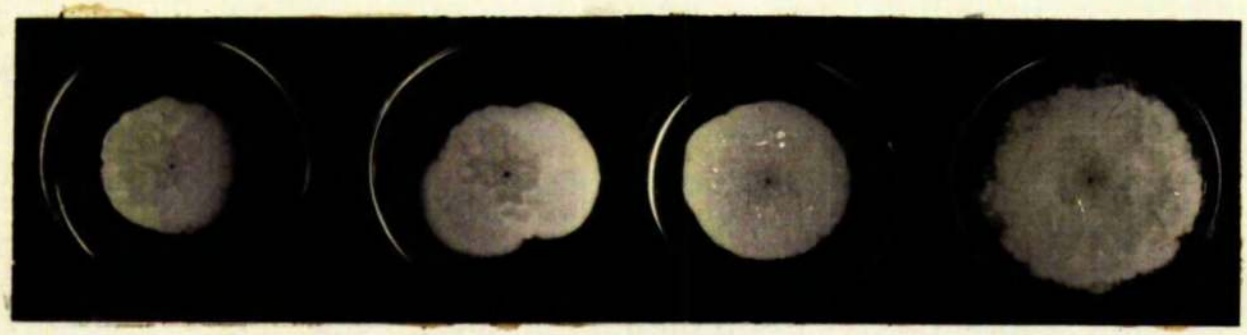
The other two variants were unstable; single sporangium cultures of each frequently changed to give rise to the other. He also observed that "saltation" was less frequent in one variant when cultures were kept growing vigorously but tended to "split" when staling took place.

A number of spontaneous variants of Phytophthora cactorum derived from the same single zoospore culture were described by Stamps (1953). Some variants remained "fairly stable" during asexual reproduction by zoospores while others segregated to give a number of different classes of progeny. Examination of the sexual progeny of four variants showed that the parental morphology could be transmitted through the oospore but that reversion and further variation were likely to occur. Old cultures were found to have increased variability.

More recently Buddenhagen (1958) investigated the variation induced in P. cactorum by ultraviolet irradiation. Of eight single zoospore colonies with changed morphology five were stable but the asexual progeny of the remainder segregated giving a small proportion of dwarf or large colonies. Dwarf "mutants" were found to sector and segregate during asexual reproduction to give faster growing forms.

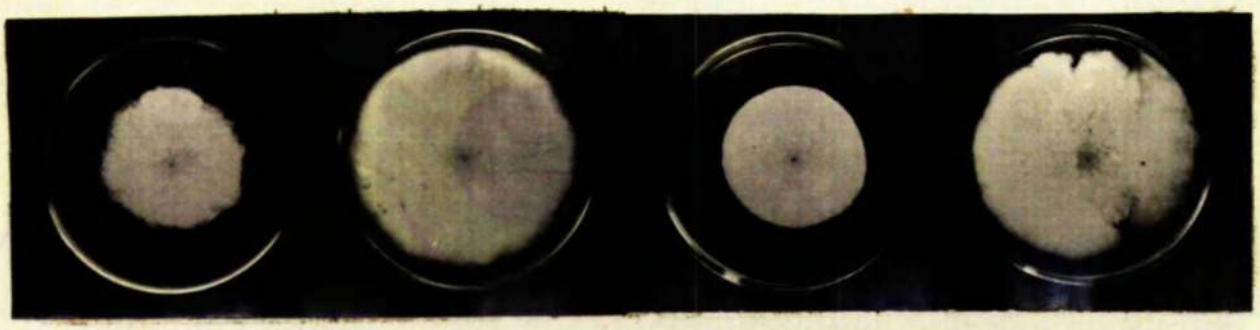
Spontaneous segregation of morphological characters was noted in Phytophthora infestans by Clarke (1964). A single zoospore isolate gave rise to compact and diffuse classes of asexual progeny. On further zoospore platings the diffuse isolates were stable whereas the dense isolates segregated again to give dense and diffuse phenotypes.

Expression of these characters was found to be influenced by the concentration of pectin in the medium.



Wild-type colony | Sectoring w.t. colony | Regular colony | Fast growing colony

Figure 22b. Spontaneous variants of wild-type.



Wild-type colony | Fast growing colony | Regular colony | Sectoring colony

Figure 23. Spontaneous variants of Sr.

Spontaneous morphological variation in P. castorum

The original wild isolate (IMI. 21168) which had been cultured for many years on oatmeal agar slopes, frequently gave rise to sectors with differing morphologies when grown on S.M.A. The single zoospore isolate used throughout this study and previously referred to as "wild-type" was found to have a stable morphology during vegetative growth and asexual reproduction. Occasionally morphologically distinct segregants were produced as sectors or as single zoospore colonies. Figure 22 illustrates some of the spontaneously occurring variants of the wild-type strain. The Sr strain similarly gave rise to spontaneous variants with a low frequency (Figure 23).

One particular culture of the wild-type isolate was found to give rise to a proportion of dwarf colonies in its asexual progeny. Of 302 colonies arising from zoospores plated on S.M.A., seven were very compact, having a growth rate of approximately half that of wild-type. Three of these dwarf colonies (dw_1; dw_2; dw_3) were isolated and transferred to plates of pea-meal agar. The dw_2 isolate had a wild-type growth rate while dw_1 and dw_3 had a considerably reduced growth rate on the pea-meal agar. The asexual progeny from each isolate was scored for the number of dwarf and wild-type colonies and the results are shown in Table 21. The progeny of dw_2 were all indistinguishable from wild-type. It seems that during growth on pea-meal agar a reversion had taken place.

	Phenotypes of asexual progeny		
Isolate	dwarf	wild-type	Percentage dwarf
wild-type	7	295	2.32
dw_1	12	144	7.69
dw_2	0	150	0
dw_3	11228	2	99.84

Table 21. Inheritance of dwarf phenotype in asexual progenies.

A high proportion of dw_1 progeny were wild-type but the frequency of dwarf phenotypes was still muchhigher than in the original progeny of the wild-type isolate. dw_3 gave rise to predominantly dwarf phenotypes but the ability to produce wild-type colonies had not been lost. It is obvious then, that three dwarf isolates differed in their ability to transmit the "mutant" phenotype to their asexual progeny.

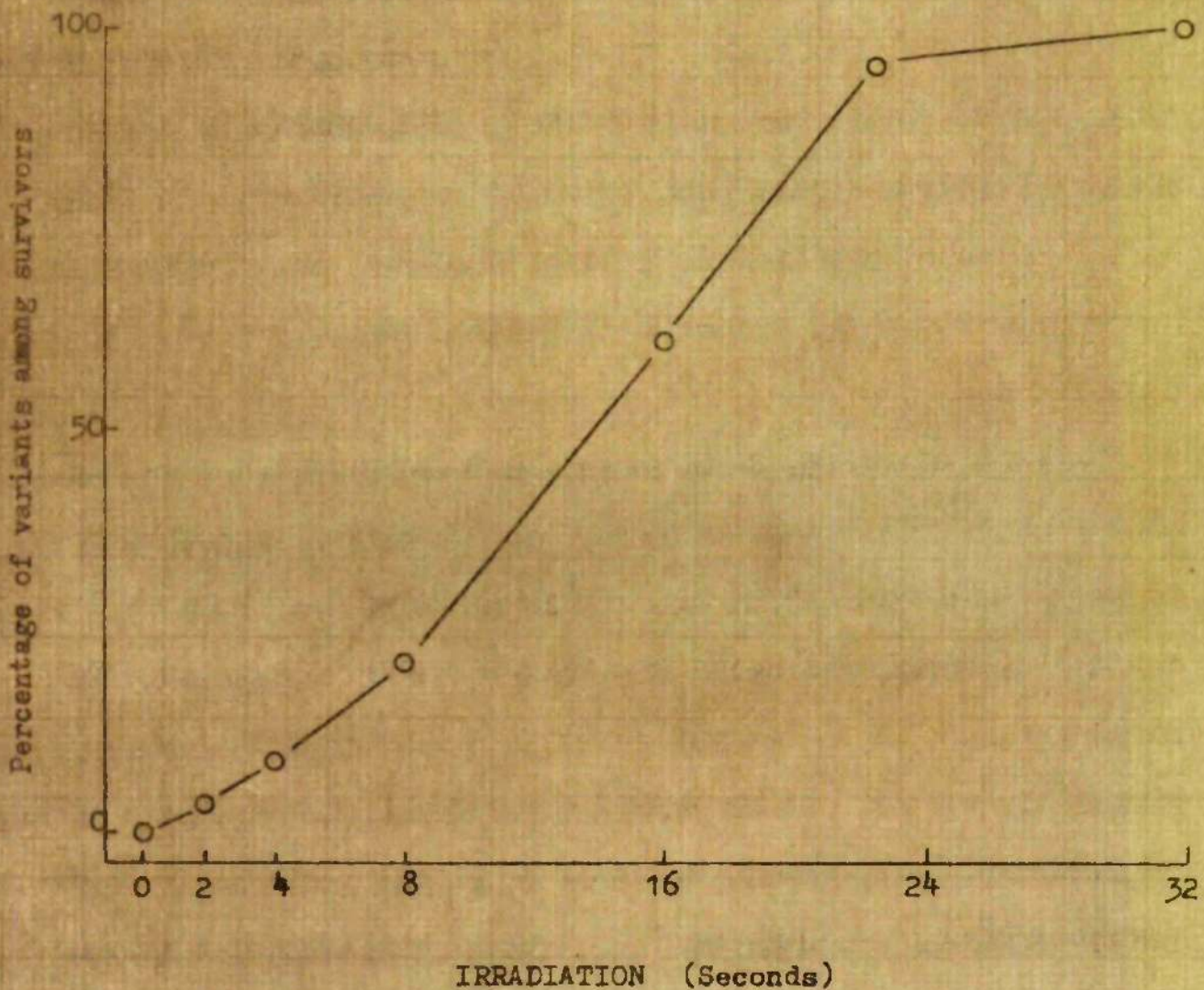
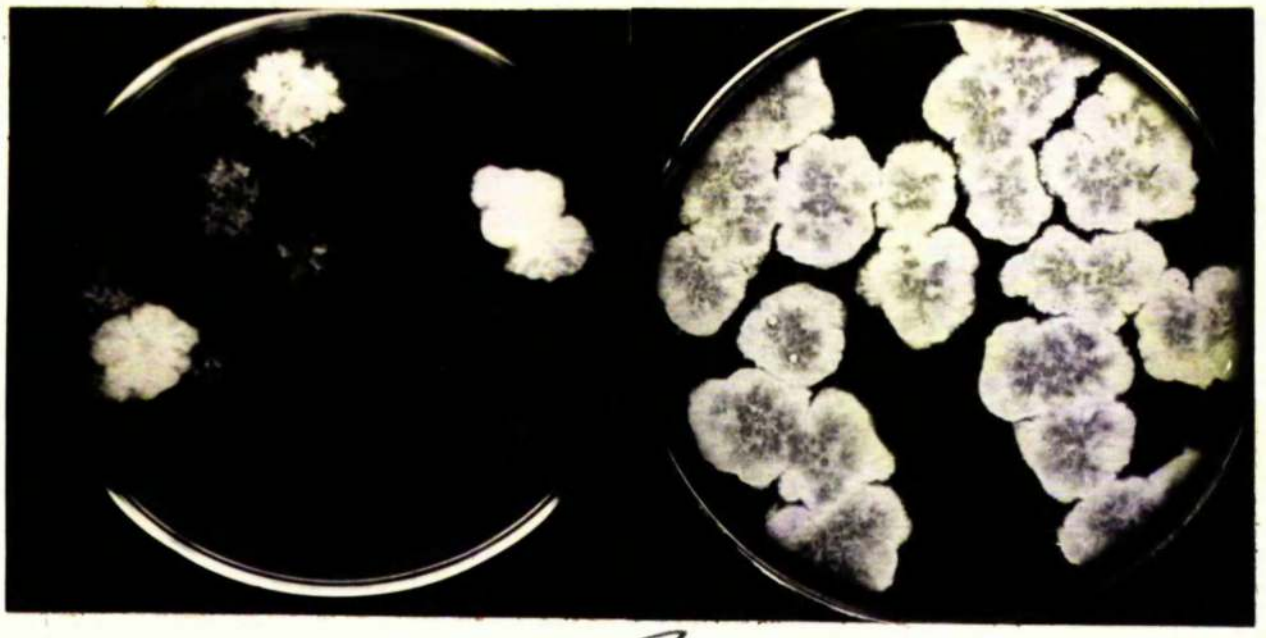


Figure 24. The relationship between UV dose and morphological variation in the survivors.

Morphological changes induced by ultraviolet irradiation

Samples of a wild-type zoospore suspension were treated with UV light for various lengths of time as described on page 62 and were plated, after suitable dilution, onto S.M.A. Six replicate plates were spread with 0.2 ml of a 1 in 100 dilution of spores treated for 0, 2 and 4 minutes respectively and six replicates were spread with 0.2 ml of a 1 in 10 dilution of spores treated for 8, 16, 24 and 32 minutes respectively. The proportion of survivors with obvious changes in morphology was noted after 72 hours incubation. Figure 24 shows the numbers of variants, expressed as a percentage of the number of survivors, plotted against time of treatment. An example of the morphological changes induced by 16 seconds irradiation is illustrated in Figure 25.

Inocula from a sample of 20 variant colonies which appeared on plates spread with spores irradiated for 16 seconds were transferred to plates of S.M.A. After ten days it was found that 13 of the growths were not obviously different from wild-type control colonies. The remaining seven showed a range of morphological variation.(Figure 26). Most of the variant growths showed segregation into sectors of differing morphologies. Slow growing colonies frequently gave rise to fast growing sectors. So great was the range of variation that it was found to be impossible to classify the morphologies of the original variant zoospore colonies or the segregants derived from them during vegetative growth. No further studies of UV induced changes were made.



Zoospores untreated

Zoospores irradiated 16 secs.

Figure 25. Variant colonies from irradiated zoospores.

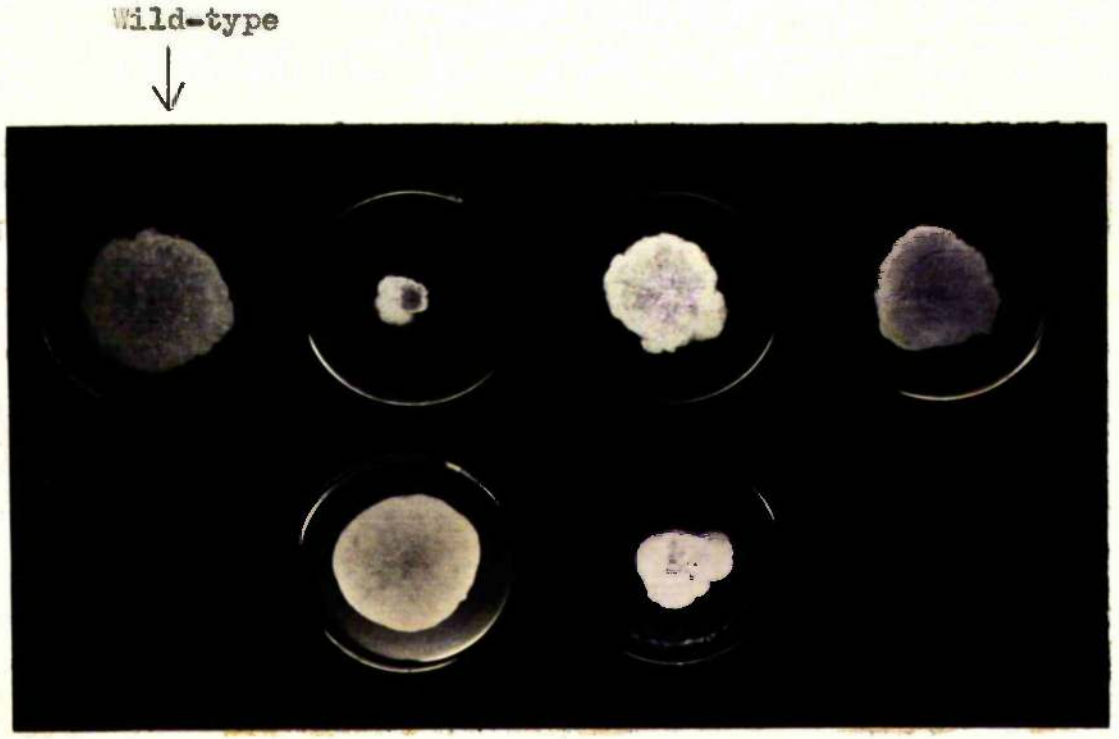


Figure 26. Sub-cultures from variant colonies.

Morphological variation induced by streptomycin

The influence of short exposures of zoospores to streptomycin.

In the screening experiments (page 66) where resistant growths were selected in media containing 100µg/ml streptomycin it was noted that survivors frequently had a changed morphology. It was decided to determine if short treatments of zoospores with high concentrations of streptomycin could induce such changes. As wild-type zoospores are sensitive to drug concentrations of 100µg/ml or more, zoospores of the resistant strain, Sr, were used. This strain has a wild-type morphology and only rarely gives rise to spontaneous variants. A zoospore suspension containing 1000µg/ml of streptomycin was prepared by adding 1 ml of a 'sucrose-salt' solution containing 10 mg streptomycin to 9 ml of a zoospore suspension (approximately 1.5×10^4 spores/ml). A control suspension had 1 ml of 'sucrose-salt' solution added to 9 ml of suspension. Samples of the treated suspension were removed after 10, 30 and 85 minutes respectively and were diluted by 1 in 10 twice. 0.2 ml samples of the 1 in 10 dilution were spread on S.M.A. So that all plates would contain the same low concentration of streptomycin, those spread with the 1 in 100 dilution of the treated suspension had been previously spread with 0.1 ml of a solution containing 0.18 mg streptomycin. Plates receiving dilutions of the control suspension were spread with 0.1 ml of a solution containing 0.2 mg streptomycin. Thus all plates contained 0.2 mg or approximately 15µg/ml of streptomycin.

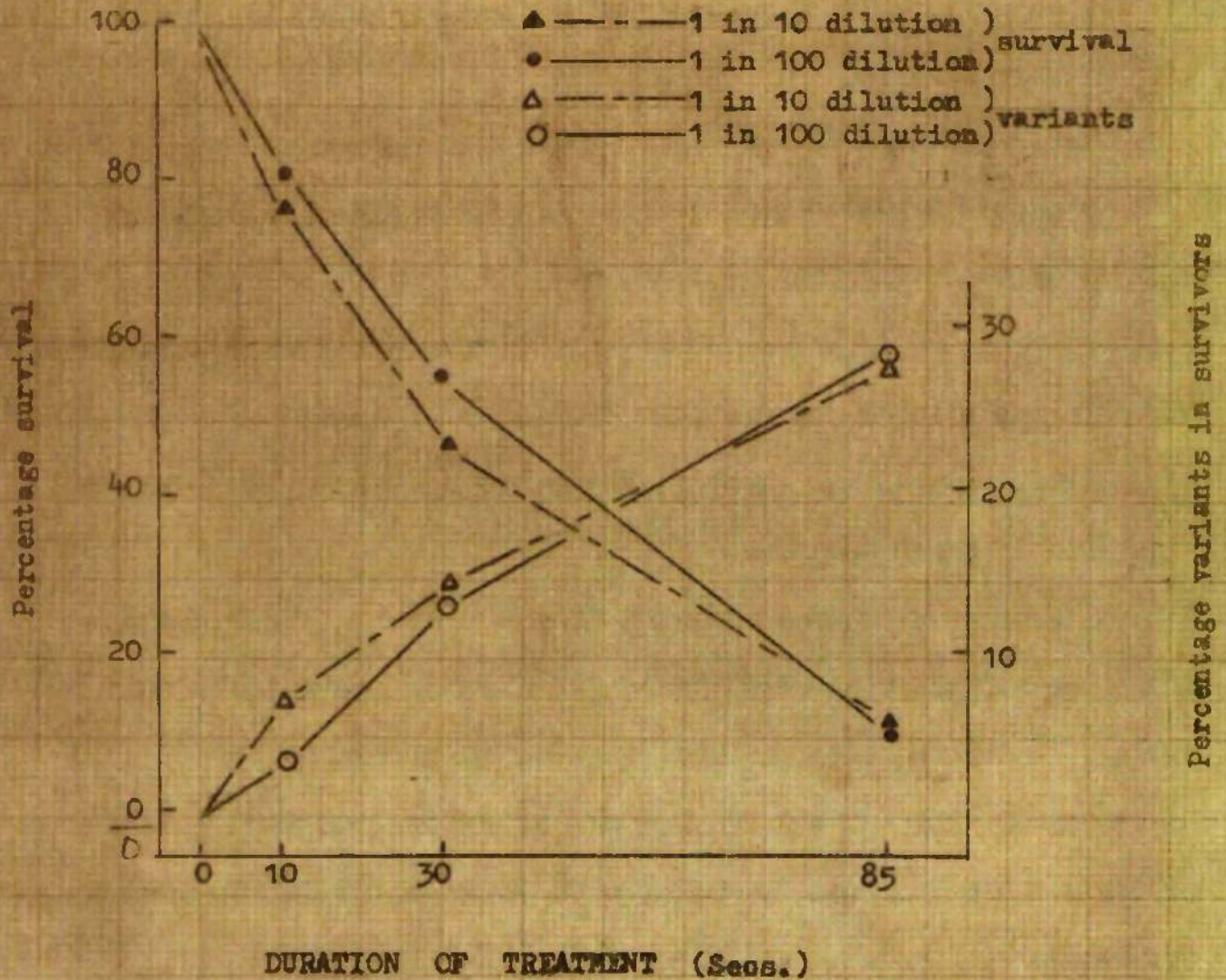


Figure 27. The survival of zoospores and the proportion of variants after various periods of treatment with Streptomycin.

Six replicate plates were spread with 0.2 ml of the 1 in 10 dilution at each time and 12 with the 1 in 100 dilution. Twenty plates were each spread with 0.2 ml of the 1 in 100 dilution of the control suspension to determine the frequency of spontaneous changes in morphology. Plates were incubated 72 hours when the number of developing colonies and the number of colonies, whose morphology differed significantly from that of the control colonies, were scored. All 417 colonies which developed in the control platings were of wild-type morphology.

The mean number of colonies per plate was expressed as a percentage of the mean number per control plate at each dilution and plotted against time of immersion (Figure 27). The number of variants in each dilution was expressed as a percentage of the number of survivors and was also plotted against time of immersion (Figure 27). Figure 28 illustrates some of the changes in morphology induced by 85 minutes treatment with streptomycin.

Sub-cultures of variant colonies grown from 1 mm disc inocula, grew into colonies not significantly different from wild-type on S.M.A. Inocula were taken from five variant colonies derived from zoospores treated for ten minutes and five from zoospores treated for 85 minutes and were transferred to pea-meal discs. Asexual progeny from these ten isolates and from an Sr isolate derived from an untreated zoospore were compared. Approximately 60 spores of each isolate were spread on each of five replicate plates of S.M.A. Colonies

Colonies from untreated zoospores.

Colonies from zoospores treated 85 minutes with 1000µg/ml streptomycin.

Figure 28. Variation induced by streptomycin.

which developed after 72 hours were examined for morphological differences. Out of approximately 150 colonies of each isolate examined none were found to be obviously different from the usual wild-type morphology of Sr. It must be concluded that the changes induced by streptomycin in this experiment were of a temporary nature and were not inherited by the asexual progeny.

The influence of inhibitory concentrations of streptomycin in the medium on the morphology of surviving zoospore colonies.

When approximately 5×10^5 wild-type zoospores were added to liquid standard medium containing 100µg/ml streptomycin in an attempt to recover drug resistant mutants (see page 66), most of the zoospores were inhibited but of the eight surviving zoospore colonies which developed, seven had a unique colony morphology when transferred to plates of S.M.A. These colonies frequently gave rise to morphologically distinct sectors during vegetative growth and the first generation ofasexual progeny from each isolate was very variable. In some instances it was impossible to classify the variety of colony morphologies found in the progenies. From the evidence available it is not possible to tell if these heritable morphological changes were in fact induced by streptomycin as the possibility that they may have been spontaneous variations merely selected in the drugged medium has not been excluded. The variation shown by one of these strains, Sd, will be described in some detail.

Morphological variation in the streptomycin dependent strains

The streptomycin dependent strain was chosen for further study because its variants were particularly easy to classify, showed an interesting pattern of segregation and did not continually give rise to novel phenotypes. There was also the possibility that the streptomycin requirement, being stable, might prove to be a useful nuclear marker with which segregation could be compared.

The strain was grown on S.M.A. + 100µg/ml of streptomycin unless otherwise stated. The first generation of zoospores of the original dependent isolate produced from mycelium grown on plates of pea-meal agar + 100µg/ml streptomycin gave rise to three distinct colony morphologies.

1) "plumulose" or "p". This is a dense colony, morphologically indistinguishable from wild-type. Young colonies from single zoospores have an irregular outline due to the formation of small "sectors" or plumes. The linear growth rate is similar to the wild-type and aerial mycelium is not abundant.

2) "knotted" or "k". In this class the colony is diffuse, has long leading hyphae which bear short, proliferating, knotted, lateral branches. Linear growth rate is faster than wild-type and aerial mycelium is absent.

3) "regular" or "r". Young colonies have a distinct and circular outline and are soon covered by a dense felt of aerial mycelium. Linear growth rate is similar to wild-type.

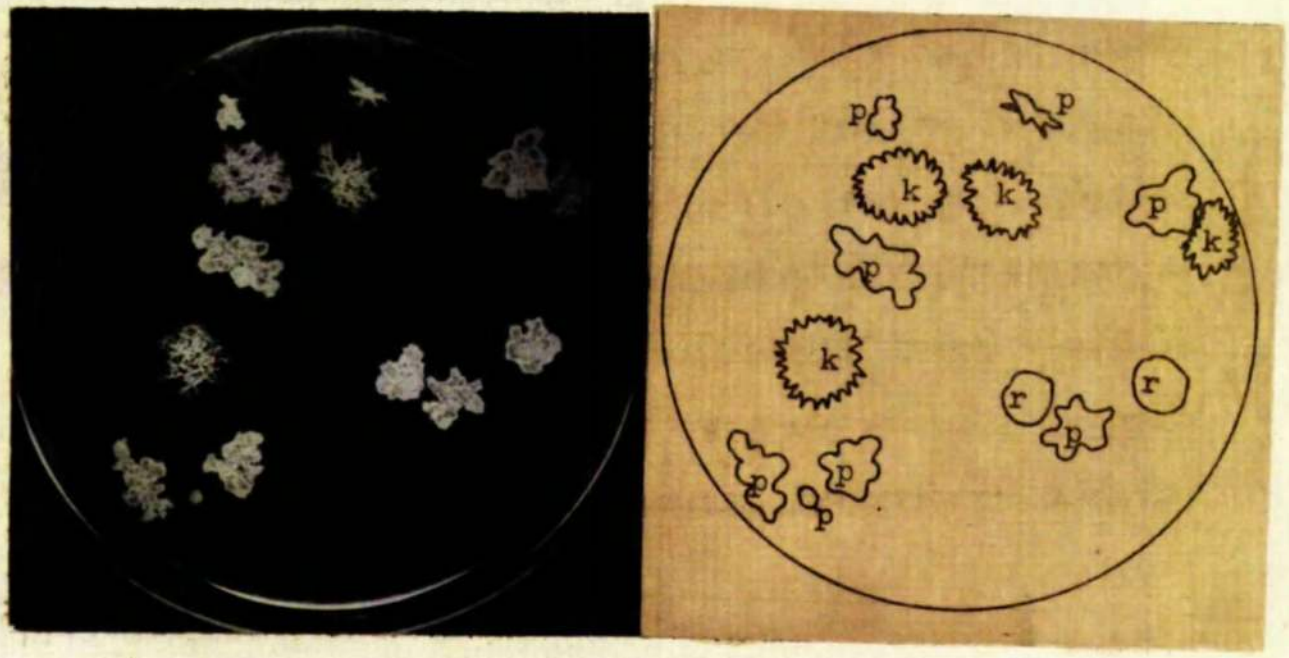
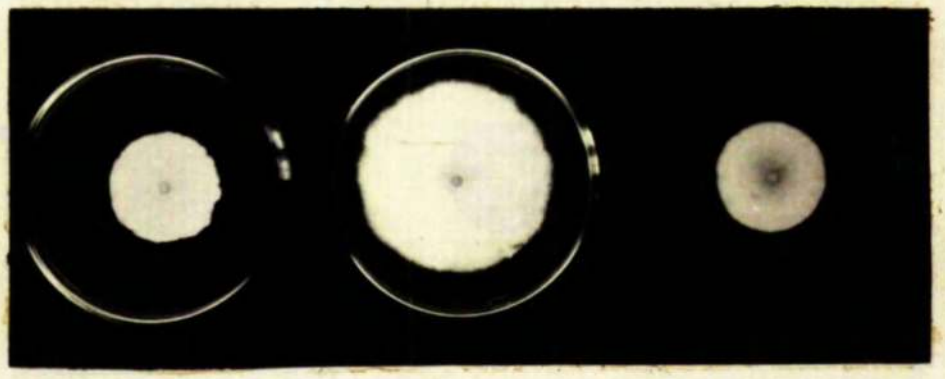


Figure 29. Plating of Sd zoospores showing the three phenotypes



p k r

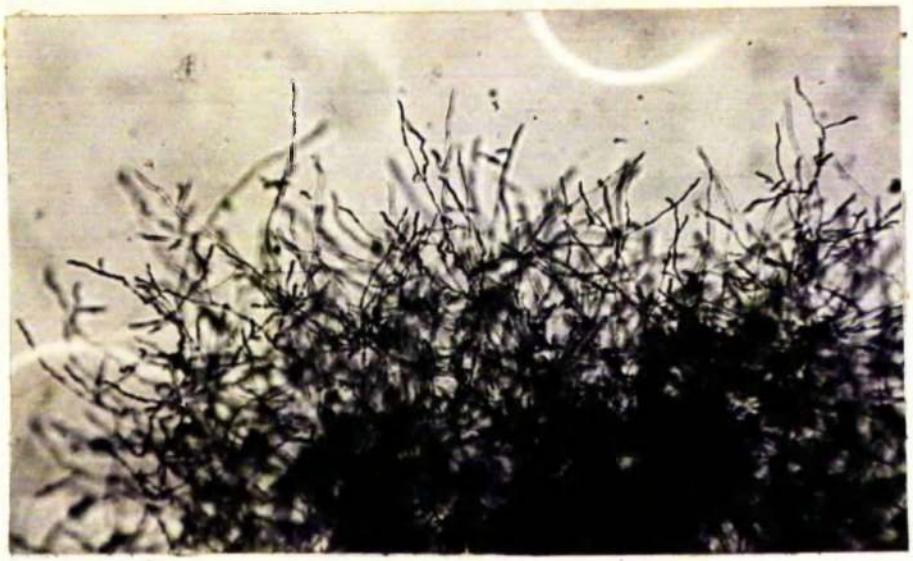
Figure 30. Eight-day old sub-cultures of Sd variants.

The three variant colonies as they first arose in the asexual progeny of the Sd isolates are shown in Figure 29. Eight-day old sub-cultures of each phenotype are illustrated in Figure 30. Differences in hyphal morphology can be observed in Figure 31. To determine the linear growth rate of each isolate, the diameter of four replicate colonies of each isolate growing from disc inocula on S.M.A. was measured at two or three day intervals. The calculated growth rates are shown in Table 22. The mean diameter of each variant was plotted

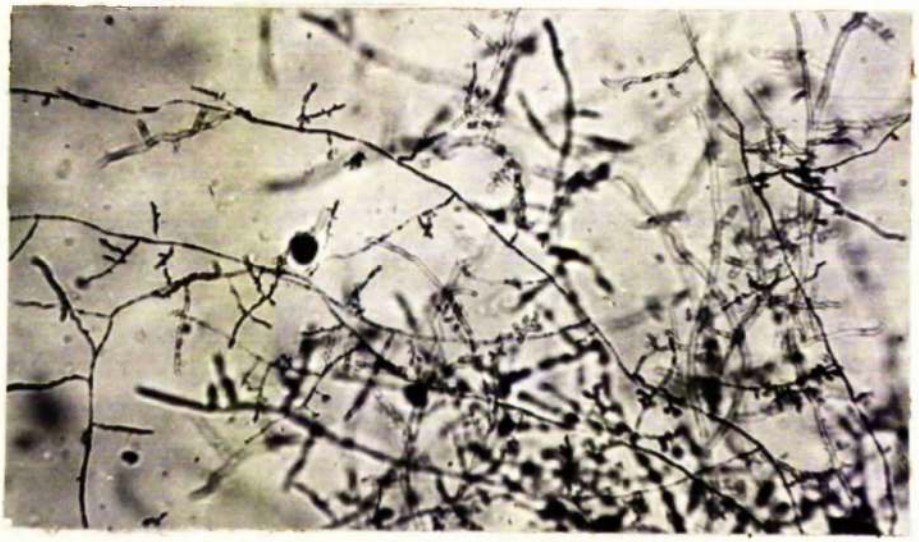
Morphology	Replicates	Linear Growth Rate mm/day	Mean Growth Rate
p	1	3.55	3.76
	2	3.52	
	3	4.22	
	4	3.74	
k	1	6.74	6.81
	2	6.80	
	3	6.72	
	4	6.98	
r	1	3.51	3.94
	2	4.03	
	3	4.18	
	4	4.05	

Table 22. Calculated growth rates of p, k and r phenotypes.

against time and the best fitting straight lines representing the



p



k

r

Figure 31. Hyphal morphology of Sd variants.

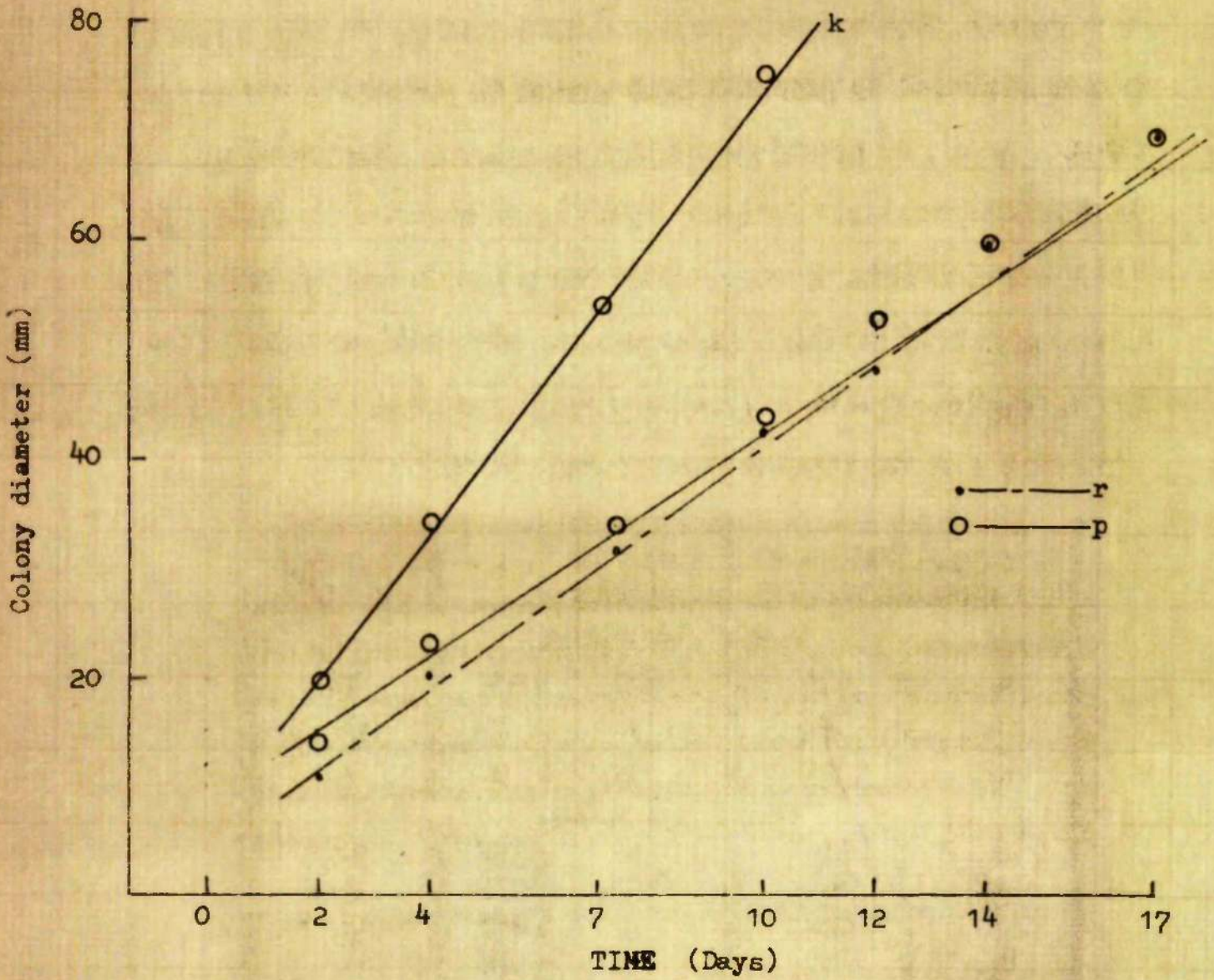


Figure 32. Calculated linear growth rates of p, k and r.

linear growth rates were calculated and are shown in Figure 32. It can be seen that the linear growth rates of p and r are not significantly different. The proportion of the different phenotypes in the first generation of asexual progeny was not determined accurately as the k and r variants had not yet been recognised as distinct. However, out of 136 colonies, 90 were of p phenotype, i.e. 66.2%.

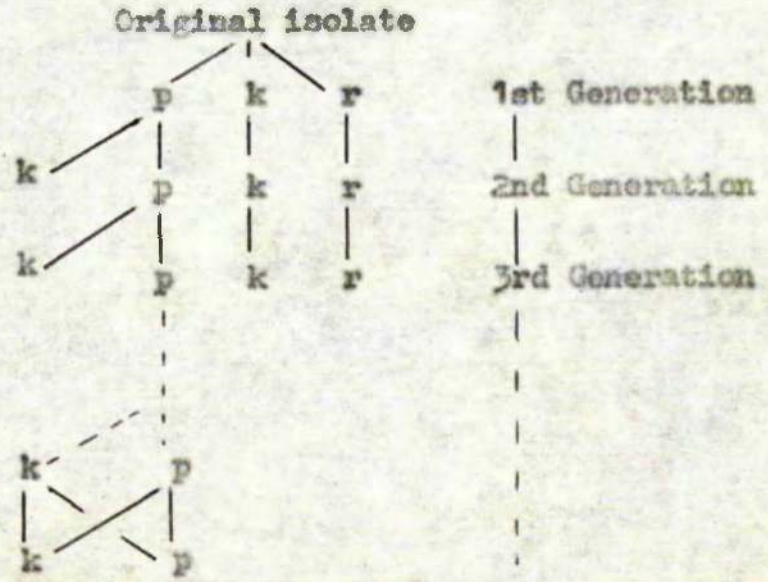
The inheritance of morphological characters in the asexual progenies.

Inocula from single zoospore colonies of p, k and r phenotypes were transferred to pea-meal agar plates to provide mycelium for the production of second generation zoospores. The phenotypes of the second generation zoospore colonies is recorded in Table 23. Two p colonies from the progeny of the p parent and two colonies from the progeny of the k and r parents were used as inocula for the production of a third generation of zoospores. Colonies were classified and the results are recorded in Table 23.

Phenotype of parent	Number of parentals	Number of segregants	Total	Percentage k in total
Second Generation				
p	169	112	281	39.9
k	84	0	84	100
r	96	0	96	0
Third Generation				
p_1	198	125	323	38.7
p_2	904	452	1356	33.3
k_1	181	0	181	100
k_2	220	0	220	100
r_1	612	0	612	0
r_2	369	0	369	0

Table 23. Phenotypes in the second and third generations of asexual progeny of Sd variants.

It is clear that r is a stable phenotype inherited by all asexual progeny. Cultures of r stored under mineral oil in oatmeal agar slants have remained stable for 18 months. Reversion to wild-type morphology has never been observed. Asexual progeny from the original k isolate are uniformly k in phenotype. During growth and asexual reproduction p colonies derived from single uni-nucleate zoospores always segregate to give k phenotypes. p colonies growing on S.M.A. not infrequently give rise to faster growing sectors of a k phenotype. It was later found that k segregants from p cultures give rise to a proportion of p phenotypes in their asexual progenies. k colonies have not been observed to sector but as k is the fastest growing variant, any slower growing sectors would be engulfed by the adjacent mycelium. The inheritance of the various phenotypes in asexual progenies is summarised in the diagram below.



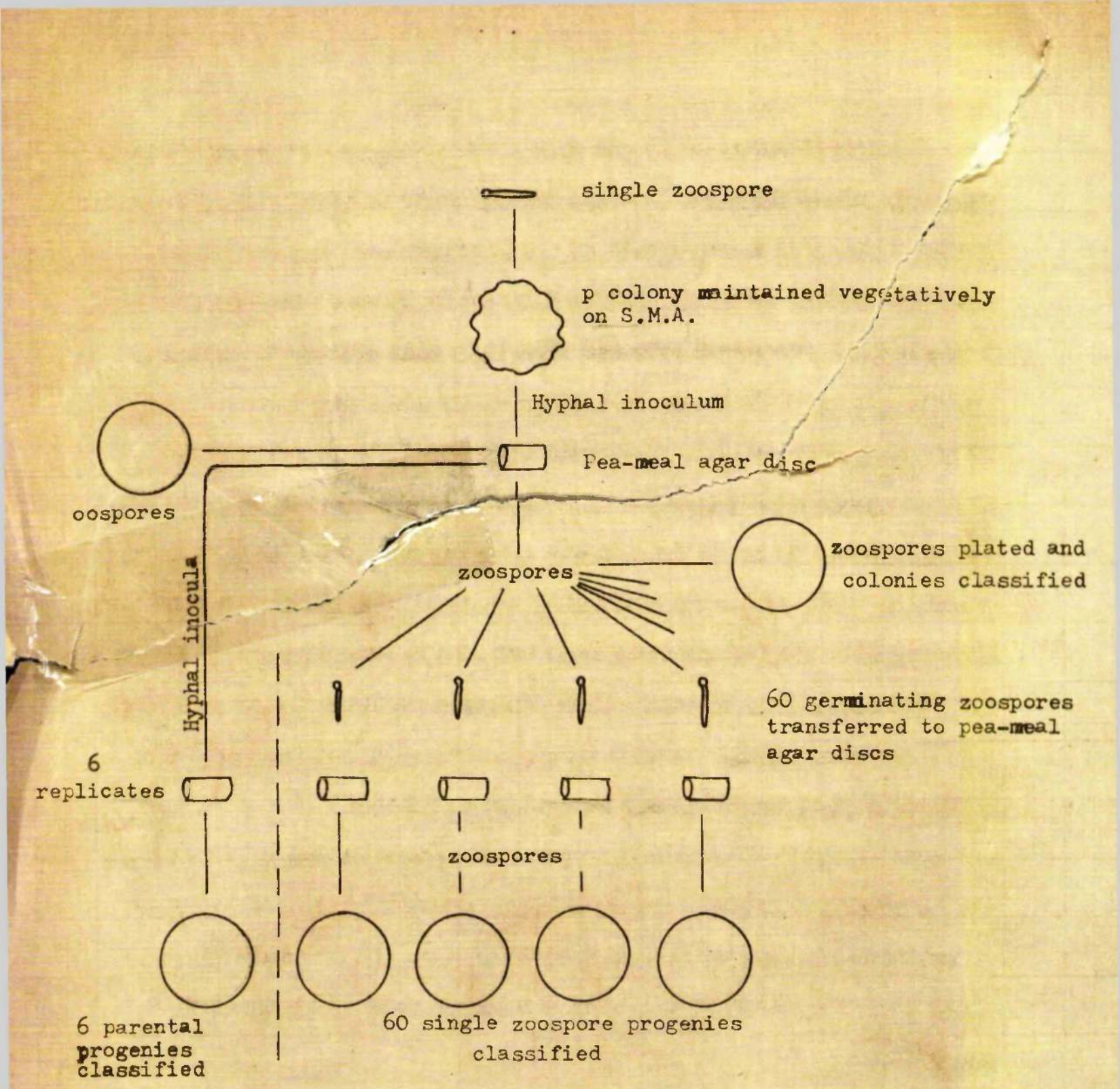


Figure 3. Method used to produce the 60 single zoospore progenies.

A more extensive study was made to determine whether zoospores from a p colony differed in their potentiality to segregate by comparing the proportion of k segregants in their progenies. A pea-meal agar disc was inoculated with mycelium from a single zoospore colony of p phenotype. Zoospores released from this disc culture (see page 31 for method) were plated on S.M.A. and after six hours incubation 60 single germinated zoospores were isolated. The plates spread with zoospores were incubated further and the proportion of segregants recorded after 72 hours (viz. 167 p colonies and 98 k colonies, or 36.9% k). Each of the 60 germinated zoospores was transferred directly to a separate disc of pea-meal agar and at the same time, six discs were inoculated with mycelium from the parent culture growing on the pea-meal disc. Zoospores were in due course obtained from all disc cultures except one which was contaminated. Samples of 0.2 ml of a suitable dilution of each suspension were spread on six plates and the resulting progenies were classified after 72 hours. The number of colonies per progeny ranged from 14 to 272, the average being about 140. Figure 33 illustrates the method used to produce the progenies.

The histogram, Figure 32 shows the numbers of progenies with different proportions of k segregants. In order to show the distribution close to 0 and 100% more clearly the proportions were transformed to angles and grouped in 5° interval classes. It can be seen that the zoospore progenies fall into at least three classes.

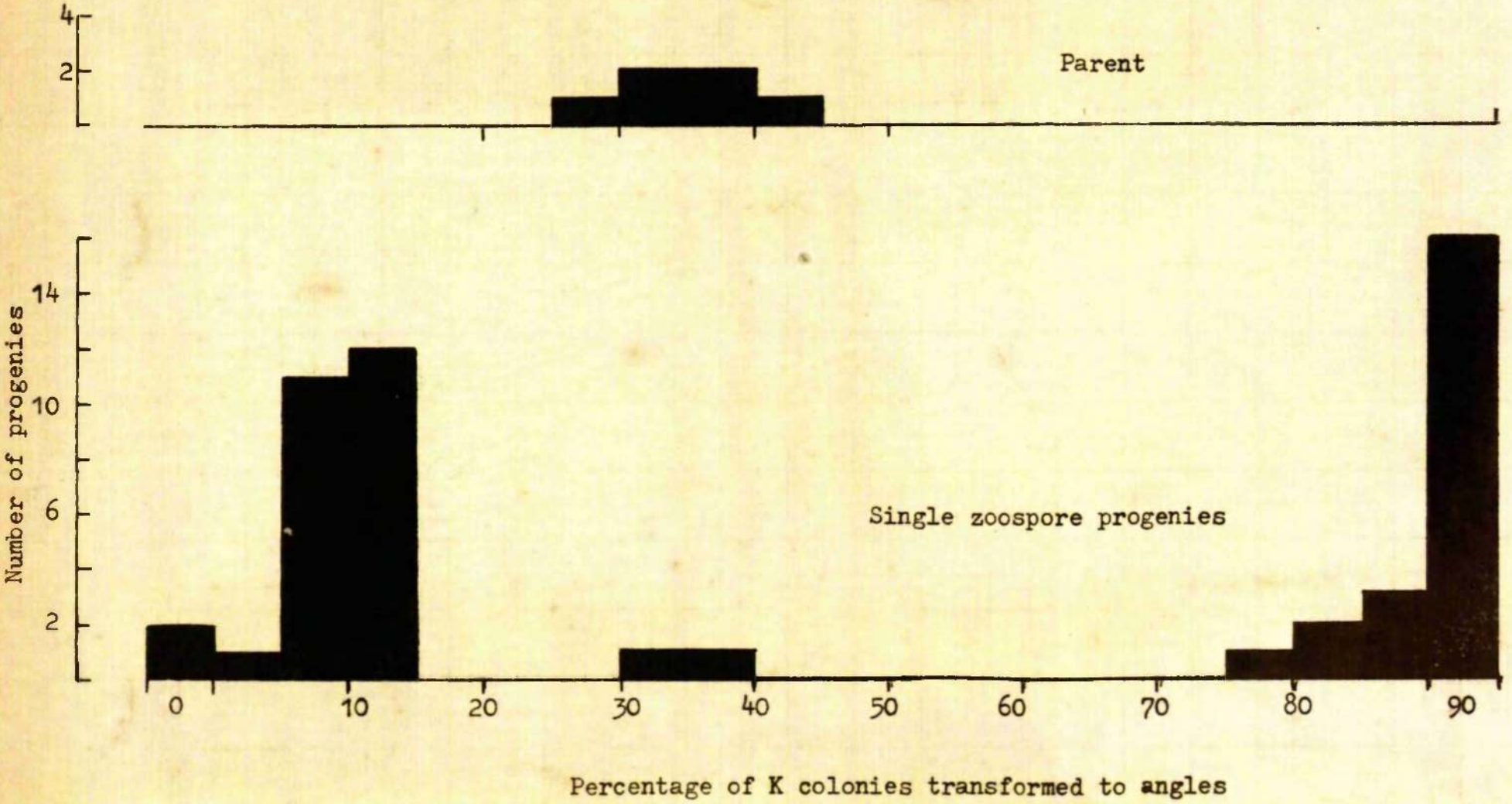


Figure 34. Number of progenies having different proportions of K segregants.

Figure 35. K segregants in a class 1 progeny.

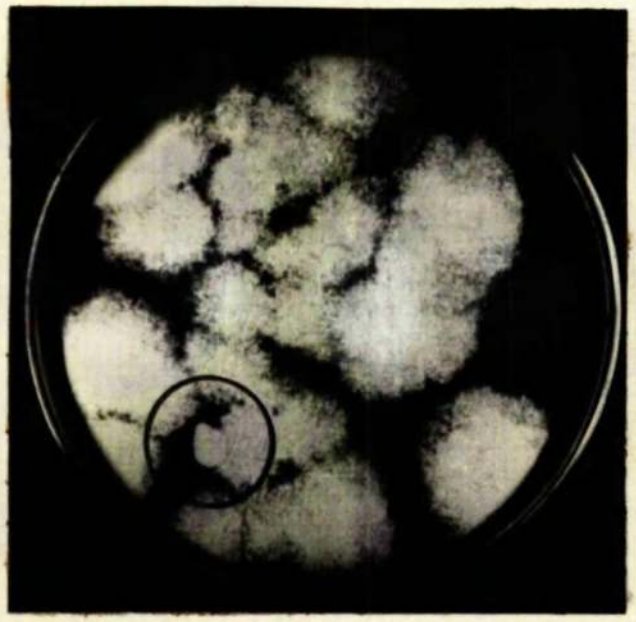


Figure 36. A p segregant in a class 2 progeny.

Class 1. Twenty-five of the progenies were predominantly p but included up to 6% k segregants.

Class 2. Twenty-two were predominantly k but gave rise to up to 5.2% p segregants.

Class 3. Only two of the progenies had significantly higher proportions of segregants. The percentages of k colonies were 30 and 33 respectively. This is within the range of the percentage of k phenotypes in the parental progenies which varied from 22 to 43.

Figure 35 shows k segregants in a class 1 progeny and Figure 36 a p segregant in a class 2 progeny. The proportion of the 60 progenies having predominantly k phenotypes (viz. 37.3%) is not significantly different from the proportion of k phenotypes in the first progeny of the parent p colony (viz. 36.9%).

One can assume, therefore, that the parent zoospores of class 1 progenies would have grown into p colonies and those of class 2 into k colonies. A zoospore parent of class 3 would presumably have grown into an unstable p colony with k sectors. This type of colony has been observed occasionally. The three classes of progeny would indicate that the original suspension contained at least three types of zoospore, each with a different potentiality for future segregation.

In the above experiments the Sd strain was always grown with 100µg/ml. streptomycin. When a non-dependent, resistant strain was derived from it (page 75) this could be grown without streptomycin.

Zoospores of the later strains, produced from pea-meal agar discs with and without streptomycin were plated on drugged and drug-free S.M.A. Similar proportions of p and k colonies were found on all four sets of plates indicating that the somatic segregation was independent of the presence of streptomycin in the medium and also that segregation occurs independently of the requirement for the drug.

The effect of ageing on the proportion of segregants in the asexual progeny.

When sub-cultures were taken from cultures of p phenotype grown on oatmeal agar and stored under mineral oil at room temperature for 18 months, they were found to be of k phenotype. Also Stamps (1953) reported that ageing of certain colonies of P. cactorum increased the variability of sub-cultures from these colonies. It was decided to determine if retention of cultures in a quiescent state for different periods of time had any effect on the proportion of segregants in the asexual progeny. Pea-meal agar discs were incubated for 72 hours at 24°C. All except three discs were then placed in a refrigerator at 3°C; these three discs were transferred to non-nutrient agar to induce zoosporangia and later zoospores, which were spread on three sets of six plates. The proportions of p and k colonies were scored after 72 hours. These first three discs had not been aged at 3°C. Sets of three discs were removed from the refrigerator after ageing 4, 8, 16 and 32 days. Transfers were made to non-nutrient agar,

Item	Df	Mean Square	F
Between ages			
Linear Regression	1	122.35	6.53*
Remainder	3	62.32	3.31
Replicates	10	18.78	

Table 25. Analysis of Variance. Ageing and the proportion of segregants.

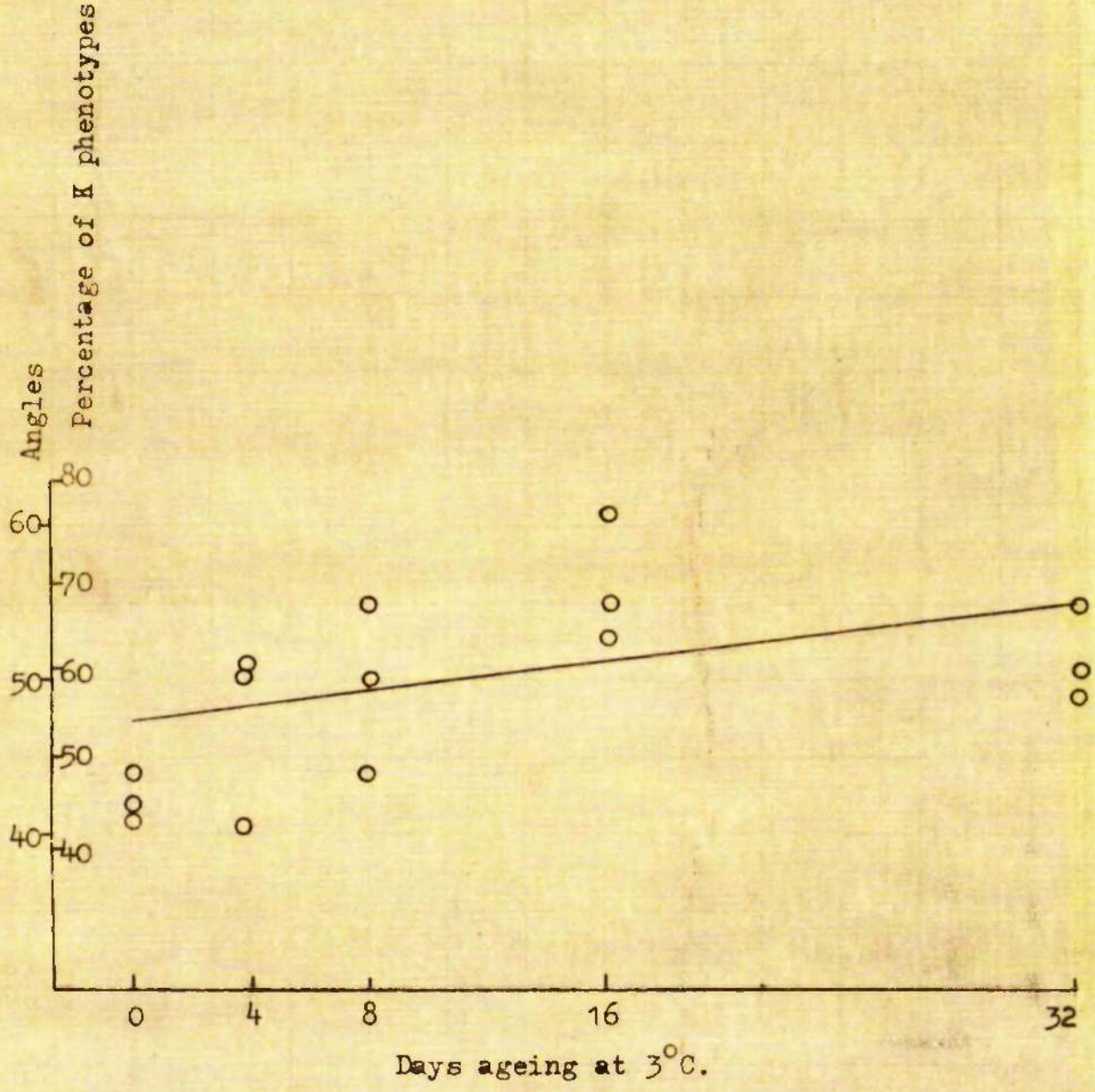


Figure 35. The relationship between culture age and the proportion of K phenotypes in asexual progenies.

zoospores were collected, plated and colonies were counted as usual. Values for the percentage of k phenotypes in the progenies, Table 24, were transformed to angles and an analysis of variance on the transformed data (Table 25) shows that the relationship between culture age and the proportion of k phenotypes in the progenies is adequately represented by a straight line. (FIGURE 35) The results may be taken to indicate that the proportion of k phenotypes in the progenies of a segregating isolate increased after storage at 3°C.

Days ageing at 3°C	Replicates	Total number of colonies	Percentage k phenotypes	Average percentage k
0	1	178	45.84	50.6
	2	232	48.71	
	3	123	44.71	
4	1	126	61.90	55.4
	2	388	43.81	
	3	195	60.51	
8	1	111	49.55	59.2
	2	198	67.78	
	3	141	60.28	
16	1	166	63.85	69.9
	2	162	77.78	
	3	194	68.04	
32	1	107	60.75	61.7
	2	114	57.02	
	3	241	67.22	

Table 24. Percentage of k phenotypes in the asexual progeny of aged cultures.

The inheritance of morphological characters in the sexual progeny.

Plates containing 1% oat-extract agar + 100µg/ml streptomycin were spread with mycelial suspensions prepared with a tissue grinder from 1) a k colony, 2) a p colony. The suspension of p hyphae was made by grinding up the pea-meal disc culture used to provide the 60 zoospore progenies analysed previously. (The percentage of k segregants in asexual progeny from this disc culture was 37.) Cultures were fed to snails when 21 days old and oospores were surface sterilized by the usual method. Single germinated oospores were transferred to S.M.A. + 100µg/ml streptomycin and the resulting colonies were classified after 72 hours. The proportions of the different classes which appeared in the sexual progeny are shown in Table 26.

	SEXUAL PROGENY				
Parent	Colonies of p phenotype	Colonies of k phenotype	Others	Total Colonies	Percentage of Segregants
p	55	11	2*	68	19.1
k	6	41	1•	48	14.6

Table 26. Phenotypes of the sexual progeny of p and k variants.

* Colonies were a mosaic of p and k sectors.

• A slow growing colony with a novel morphology.

As in the asexual progenies of these variants a proportion of k segregants appear in the sexual progeny of p and a proportion of

p segregants appear in the sexual progeny of k. The limited amount of evidence available indicates that a lower proportion of k segregants is found in the sexual progeny of p than in the asexual progeny of the same isolate (see Table 27).

	Phenotypes		
	k	p	Total
Asexual progeny	98	167	265
Sexual progeny	11	57	68
Total	109	224	333

$x^2_{(1)} = 11.01^{**}$ p very small

Table 27. The numbers of p and k phenotypes in the sexual and asexual progenies of p.

The asexual progeny from two single oospore colonies of p phenotype was examined. Both were found to contain a proportion of k segregants indicating that the ability to segregate had not been lost.

As the germinating oospores from the k culture were being picked out they were classified as large or small. Large oospores had given rise to more than one germ tube with or without terminal sporangia while small oospores had single germ tubes with terminal sporangia. The proportions of p and k colonies in the two classes of oospores

is shown in Table 28. Whether there are significantly more p segregants

	Colonies of k phenotype	Colonies of p phenotype	Others	Total Colonies	Percentage of Segregants
Large oospores	13	0	1	14	0
Small oospores	28	6	0	34	18

Table 28. The proportions of p segregants in large and small oospore classes of a k isolate.

in the culture derived from the small oospores than from the large (0 : 14 :: 6 : 28) was tested by the exact method (Fisher 1950 § 21.02, Freeman & Halton 1951). The probability of getting as great or greater deviation from proportionality as here observed, by chance, is 0.155*. Thus there is no evidence that spore size has an effect on the proportion of segregants.

* This is obtained by calculating the probability $P_x(t)$ of each array, $x(t)$ with the same marginal totals as the observed array $x(1)$, and adding all $P_x(t)$ such that $P_x(t) \leq P_x(1)$ Thus

$x(t)$	14	0	13	1	.....	9	5	8	6
	28	6	29	5		33	1	34	0
$P_x(t)$	0.109		0.317			0.045		0.001	

The influence of zoospore size on segregation in the asexual progeny.

On the other hand, zoospore size was found to have an effect on segregation. A zoospore suspension from a p culture, containing a range of spore sizes was produced using cholesterol as described on page 32 and was plated on S.M.A. After six hours single germinated spores were isolated and classified in one of three size classes. Forty spores less than 14μ, 40 between 14 and 20μ and 40 more than 20μ in diameter were isolated. Phenotypes were recorded after 72 hours.

A proportion of intermediate phenotypes between p and k were observed in the 14μ to 20μ class and in the 20μ class. Only typical k type segregants were scored. Table 29 shows the percentage of k segregants in each size class. It is obvious from the results that the proportion of k segregants decreases with increasing size of zoospores.

Zoospore Size (Diameter)	Total Colonies	Colonies of k phenotype	percentage k segregants
20μ	39	5	13
14μ - 20μ	40	18	45
14 μ	40	28	70

$x^2_{(2)} = 26.48$ p very small

Table 29. The percentage of segregants in the three size classes.

Discussion

Any explanation of morphological variation in *P. castorum* must be consistent with the following facts.

1) Variation occurs spontaneously or may be induced.

2) The changed phenotypes may be inherited or reversion to wild-type may occur.

3) Characters may segregate persistently during vegetative growth and asexual reproduction.

4) Somatic segregation is influenced by spore size and ageing.

5) Segregation is apparent in the sexual progeny of the strains exhibiting somatic segregation.

As pointed out previously, morphological differences may arise primarily by a change in the nuclear genetic material or by extra-nuclear changes. Changes in phenotype which are heritable must be under the control of some self-replicating or self-perpetuating system. An explanation could therefore be based on such a system located within the nucleus or in the cytoplasm. Jinks (1963) has enumerated seven criteria which characterise extrachromosomal systems, viz.

1) Differences between reciprocal crosses or uni-parental transmission of the character.

2) Non-mendelian segregation. For this test to be valid the behaviour of nuclear markers must be observed simultaneously to ensure that the segregation in question is not due to abnormal

nuclear behaviour during reproduction.

3) The heterokaryon test (Jinks 1954). The segregation of known nuclear markers in a heterokaryon is compared with the segregation of the suspected cytoplasmic factor.

4) Infection. Where differences can be transmitted through a mature mycelium and where nuclear migration can be ruled out, invasive spread of cytoplasmic determinants is indicated.

5) Persistent segregation in homokaryons. Segregation in the asexual progeny derived from a single uni-nucleate haploid spore suggests a segregation of cytoplasmic factors.

6) Mutation. High mutation rates and the induction of specific changes are evidence for extrachromosomal heredity.

7) A change in cytoplasmic structures correlated with a change in phenotype. A detectable change in a cytoplasmic element possessing genetic continuity would lend support to a claim for extrachromosomal control.

The more of these criteria that can be satisfied, the stronger is the evidence for the operation of a system or systems of extra-chromosomal heredity. But before cytoplasmic inheritance can be evoked it is necessary to exclude all possible ways in which changes in the nuclear genetic material may be responsible for variation.

Explanations of the observed variation based on nuclear models.

The somatic segregation in the Sd strain

1. Somatic segregation can occur when mitotic crossing over takes place in heterozygous diploid nuclei but homozygous recombinants are produced which would not segregate further unless the heterozygous condition were regenerated by mutation. However, if the variation were controlled by multiple loci and the segregating strain were heterozygous at a number of these loci it is conceivable that segregation could continue by further crossing over. The frequency of mitotic crossing over found in fungi, e.g. of the order of 10^{-3} per conidium in Aspergillus (Pontecorvo et al. 1954) could hardly account for the observed segregation.

2. Examples are known in higher plants where the presence of a certain mutant allele in a genome causes a high frequency of specific mutation to occur at another unlinked locus (Brink 1960). A heterokaryon would be formed if nuclei bearing paramutable and paramutagenic loci were present in a mycelium. Homokaryons would become heterokaryotic as soon as the paramutable locus mutated; they could then segregate into differing homokaryons.

3. If the cytoplasm of the segregating strain contained, instead of nuclei with different genotypes, nuclei of different ploidy, segregation could give rise to homokaryotic individuals with one or the other ploidy only. Haploid nuclei might determine one phenotype and diploid nuclei the other. To account for the observed frequency of persistent

segregation, a change in ploidy would require to take place frequently during growth, i.e. haplodization and fusion. Such a system could result from a mutation of a gene regulating ploidy change. Although heterokaryons have been observed with haploid and diploid nuclei (e.g. Takeichi & Ikeda 1961) such a balanced system with frequent ploidy changes has not been observed.

Morphological variation induced by UV irradiation

As indicated in Figure 22, 25 per cent of the survivors of 8 seconds irradiation had changed morphologies. This dose allowed approximately 40 per cent survival (Figure 9). Buddenhagen (1958) has shown that similar changes induced in P. cactorum with similar frequencies were heritable, at least during vegetative growth and asexual reproduction. Such high frequencies of induced mutation at doses of UV allowing high survival are not expected of nuclear genes. The usual frequencies observed in fungi are in the order of 1 in 10^5 survivors (Kölmark 1953). It is therefore unlikely that gene mutation could account for the induced variation

None of these models based on nuclear gene behaviour provides a convincing explanation of the observed phenomena, so we proceed to consider models based on extrachromosomal determinants.

Models of morphological variation based on extrachromosomal systems

Only a few of Jinks' seven criteria listed above can be invoked to test the extrachromosomal nature of the observed variation. Segregation from asexual uni-nucleate zoospores and from sexual oospores, in both cases independently of streptomycin dependence, has been observed, and is suggestive of a cytoplasmic system. Nuclear control cannot, however, be excluded as known nuclear markers were not present. The high frequency of mutation induced by ultraviolet light is also suggestive of a cytoplasmic system. Of the other criteria, the presence of uni-parental transmission during sexual reproduction cannot be tested as yet, since only selfed progeny are available. Also it is not possible to make a heterokaryon test in the absence of nuclear markers. In preliminary experiments in which inocula of p, k and r strains were transplanted into growing colonies of each other, no evidence of infective transmission was obtained. Cytoplasmic elements of variants have not been examined for the presence of visible changes. There is, however, no evidence to suggest that more of the criteria might not be satisfied if nuclear markers were available.

The somatic segregation in the Sd strain

1. The presence of somatic segregation in the Sd strain indicates that if controlled by cytoplasmic determinants, these are particulate in nature. An interpretation of the segregation could be based on the random distribution of particles during growth and sporogenesis. If there were many particles present in each zoospore, all of the

same kind, the mutant phenotype (k) might be manifest when the number of particles dropped below a critical level. During growth of the mutant, multiplication of the determinant could restore the number of particles in the cytoplasm to a supra-critical level allowing wild-type sectors and asexual progeny to be formed. On the other hand if few particles were present in each spore, random distribution at sporogenesis might be expected to give rise to a class of spores which lacked any particles and were therefore of mutant phenotype. However such phenotypes would not segregate further and therefore this explanation could not account for the observed p-k segregation.

2. Another interpretation could be based on a random distribution of two homologous kinds of particle, wild-type and mutant. If large numbers of particles were present in each spore, a mutant phenotype could result when a spore contained a proportion of wild-type to mutant homologues below a critical level. The wild-type phenotype could be regenerated if the ratio of wild-type to mutant particles increased during growth. Intermediate phenotypes might arise when the proportion of particles in a spore was at the critical level. On this model, populations of large and small spores from the same mycelium should both contain spores with proportions of particles above and below the critical level; also small spores would be as likely as large spores to contain the critical proportion and should therefore give rise to a similar frequency of intermediate phenotypes. Clearly this does not agree with the results obtained, viz. that small spores always

gave rise to distinct wild-type or mutant phenotypes whereas large ones gave rise to a considerable proportion of intermediate phenotypes.

3. A system based on the segregation of a small number of wild-type and mutant homologues might be considered next. Somatic segregation from a zoospore would require either that the spore contained both normal and mutant particles, or that it contained only one type of particle and the other homologue arose by mutation during growth. In the former instance the number of determinants per spore could be estimated from the pattern of segregation in the progenies of a sample of such spores. If the proportions of the two homologues in the mycelium were a and b and there were n homologues per spore, then there will be n + 1 kinds of spore in which the proportions of the two determinants are given by the terms of the expansion of $(a + b)^n$ if the determinants are included in the spores at random. These different kinds of spores would give rise to progenies which segregate with different ratios. Thus if x different kinds of progeny can be distinguished in respect of the proportions of the two morphological types, there must be x - 1 determinants per spore (Arlett Grindle & Jinks 1962).

The three classes of spore progenies which were distinguished, therefore, might seem to indicate that the zoospore contained two determinants. Assuming that approximately equal numbers of determinants were present in the hyphae prior to sporulation, the class which received both wild-type and mutant determinants should be twice as frequent as either of the classes receiving only mutant or only wild-type.

determinants. Also the latter classes would not be expected to segregate further. This is not consistent with the observed facts.

Alternatively, if a system based on one particle per spore were operative, only two classes of spore would be expected (as found) and neither would segregate further. However, such spores could give rise to segregating progeny if we assume that mutation from wild-type to mutant determinant and vice-versa occurred with a high frequency. To account for the rare class with a high proportion of segregants we must assume that occasionally two determinants are included in one spore. If both were identical the spore would be indistinguishable from one receiving a single determinant but if the determinants were different the spore would give rise to equal numbers of mutant and wild-type progeny assuming an equal rate of multiplication of each determinant, absence of selection and equal rates of forward and back mutation. The rare class could also have arisen if mutation from one homologue to the other took place soon after spore germination. The frequency of mutation and the time at which it first occurred after germination might be expected to influence the number of segregants in the progeny. The mutation frequency would need to be high enough to account for the fact that most, if not all of the progenies include both phenotypes. A cytoplasm containing both wild-type and mutant particles has been referred to as a heteroplasmon (Jinks 1959) as distinct from a cytoplasm containing different kinds of nuclei - a heterokaryon.

This model system could also account for the different patterns of segregation in spore populations of different sizes. If the normal sized zoospore contains only one determinant it is reasonable to assume that larger spores derived from protoplasm which failed to cleave into a number of smaller spores, would contain more than one determinant. A large proportion of the large spores formed from a heteroplasmic mycelium would be heteroplasmic while a smaller proportion would be homoplasmic for wild-type and mutant homologues. As the number of determinants per spore increases with the size of the spore the numbers of homoplasmic spores would be expected to decrease. The limited evidence obtained is consistent with this explanation (see Table 29). The population of normal sized zoospores gave rise to a high proportion of k phenotypes which could be taken to indicate a high proportion of mutant determinants in the heteroplasmic sporogenous hyphae. As the size class increased, fewer of the resulting colonies had a pure k phenotype (mutant homoplasmons) and an increasing proportion had an intermediate phenotype (heteroplasmons). By examining the progenies produced by spores of different sizes, intermediate phenotypes might have been resolved into proportions of typical p and k phenotypes and estimates of the numbers of determinants in spores within various size classes could have been made. A colony derived from a normal zoospore carrying only one determinant would require to become heteroplasmic before sporulation as few progenies failed to segregate; presumably the determinant mutates from wild-type to mutant and vice-versa

with high frequency.

The proportion of the different particles in the mycelium might determine the phenotype, a high proportion of wild-type determinants giving a pure p colony and a high proportion of k determinants giving a pure k colony. A continuous range of intermediate phenotypes would result from intermediate proportions of the two determinants. Presumably the equilibrium proportion of p and k, which determine the phenotype in any one environment is influenced by:-

1) The rate of forward and back mutation in that environment.

2) The relative rate of multiplication of each determinant. Certain environments may confer a selective advantage on one type of determinant and so favour an increase in the proportion of that determinant.

The higher proportions of segregants in the progenies of the 25 p isolates than in the 22 k progenies (See Figure 34) may indicate that the mutation to k or the rate of multiplication of the k determinant exceeded that of the p or wild-type determinant under the conditions of the experiment, i.e. during growth on pea-meal agar and germination on S.M.A.

It was observed that during continuous culture on S.M.A., single zoospore colonies of p phenotype gradually lose their typical morphology and become more like the k phenotype. This could be due to an increase in the proportion of k determinants as an equilibrium heteroplasmon is formed. Typical k-type sectors are also formed presumably when

only mutant determinants become segregated in a hyphal branch. The p-type morphology then can only be maintained by selection. Single zoospore colonies of k do not seem to change as much during continuous culture on S.M.A. It is possible that the equilibrium is attained on this medium in a heteroplasmon with a higher proportion of k determinants. This is understandable, as the k phenotype has a high linear growth rate on S.M.A. and will be selected. Any slower growing areas with a higher proportion of wild-type determinants or homoplasmic wild-type sectors will be engulfed.

As already stated, the original zoospore suspension of the Sd strain gave rise to three distinct colony morphologies, two of which, viz. k and r remained true to type during asexual reproduction (see page 105). The p-k segregating line discussed above was derived from a single p-type colony from the original zoospore suspension. The stable k and r phenotypes arising at the same time as the segregating line may well be determined by a similar cytoplasmic system. The absence of wild-type segregants might be taken to indicate permanent loss of self-reproducing determinants. Alternatively if mutation of a determinant occurred and wild-type determinants were absent or suppressed the wild-type phenotype could not be regained.

It is not possible to define whether the origins of the three phenotypes was spontaneous or was induced by streptomycin but it is interesting to note that this drug is able to induce a number of uniparentally inherited changes in Chlamydomonas (Sager 1962) and can

effect the irreversible loss of chloroplasts in Euglena (Provasoli 1948).

If zoospores were to contain but one homologue of a certain cytoplasmic determinant there must be some mechanism ensuring a precise distribution at sporogenesis. The determinant might be expected to be associated with a self-reproducing cytoplasmic organelle, only one of which is included in each spore. As the ultra-structure of the zoospore is unknown it is impossible to suggest where a single determinant might be sited. It is still uncertain which organelles are self-replicating and possess genetic continuity but there is increasing evidence to suggest that mitochondria, centrioles and kinetosomes are autonomous. We do know, however, that the majority of zoospores contain only one nucleus. The single determinant postulated could, then, be in the nucleus itself, perhaps a paramutable locus as suggested previously and the unstable colonies would be heterokaryotic and not heteroplasmic. The rare class of zoospores giving a high proportion of segregants could have been bi-nucleate. A small proportion of bi-nucleate spores is frequently found in a population of uni-nucleate spores but the latter are usually distinguishable by their larger size and care was taken during isolation of the 60 single germinated zoospores to select only those with a small spore.

Interpretation of segregation in the sexual progeny of the Sd strain

It was shown that both p and k phenotypes are transmitted to the sexual progeny and the ability of each to continue to segregate has not been lost. The segregation ratios of p : k in zoospore and

oospore progeny was different (see page 111). It could be that if the fungus were a diploid heterozygote, segregation of nuclear genes during meiosis affected the expression of the p and k phenotypes. On the other hand there is no evidence that karyogamy occurred in these oospores. The segregation could then be the result of the same system which determines segregation from zoospores, with the following factors perhaps affecting its incidence :-

1) The proportions of p and k determinants could be altered by different selection pressures or mutation rates during growth on pea-meal agar and oat-extract agar.

2) Selection may operate during germination so that oospores of different constitution may have had a different percentage germination.

3) Oospores are aged before germination commences.

4) Oospores are treated with a variety of biologically active chemicals within the gut of the snail and afterwards.

It is interesting to note that phenotypes of single oospore colonies were never intermediate as were a proportion of colonies derived from large zoospores of a comparable size. This might indicate that mature oospores contain only one determinant. Blackwell (1943a) found that oospores contain a single nucleus prior to germination. However if cytoplasmic determinants were involved the number present in the gametangia might be reduced during oospore formation and one type of homologue, possibly the mutant one, might be eliminated preferentially. Mather and Jinks (1958) suppose that a restandardisation

of the cytoplasm takes place during sexual reproduction. Rejuvenation of wild-type from "low sexual", "B" and "minute" variants of Aspergillus nidulans during sexual reproduction has been used as evidence in support of this hypothesis.

The possible nature of other spontaneous and induced morphological variation.

Besides p, k and r, many different spontaneous and induced variants have been observed. The high frequency of UV induced changes and the possible mutagenic action of streptomycin would suggest that these changes are cytoplasmic rather than nuclear. A model system accounting for the variety of changes observed would require to have either one site at which many different mutations could occur (analogous to multiple alleles at one locus) or many sites at which one or several changes could occur. The high frequency of induced variants resembles the high frequency of induction of temperate phage in bacteria by UV (Lwoff & Gutmann 1950). It has been shown also, that episomes occur which may function as cytoplasmic factors or may become integrated into the bacterial chromosome (Jacob & Wollman 1958). Although temperate viruses or episomes have never been demonstrated in fungi it is possible that similar particles exist and may become independent cytoplasmic determinants spontaneously or by induction.

Of the various possibilities suggested to account for morphological variation in P. cactorum a model based on the existence of heritable cytoplasmic particles capable of mutation seems to be the most reasonable.

It is obvious that segregating nuclear markers are required before further definitive tests can be made. Only when these are available can the segregation observed be compared with the segregation of nuclear genes. There is also the possibility that heterokaryons might be formed allowing further criteria of extrachromosomal inheritance to be tested.

CONCLUDING SUMMARY

AND

DISCUSSION

Concluding Summary and Discussion

A reliable method of producing asexual progeny and a method of germinating oospores and establishing sexual progeny in pure culture has made it possible to analyse the inheritance of certain variant phenotypes.

The inheritance of resistance to certain drugs has been examined in some detail. Resistance to, and dependence on streptomycin was stable and did not segregate in the asexual or sexual progenies. Resistance to acti-dione and sulphanilamide was shown to be a temporary adaptation which disappeared in the absence of the drug.

Morphological variants arose spontaneously and could be induced by treating zoospores with ultraviolet irradiation or streptomycin. The inheritance of morphologically distinct phenotypes was examined in a streptomycin dependent strain. Segregation was shown to occur in the asexual and sexual progenies independently of the streptomycin requirement which did not segregate.

It was hoped that a number of nuclear markers could be obtained which would segregate in the sexual progeny and thereby indicate the ploidy of the somatic nuclei and the position of meiosis in the life-cycle. This has not been possible due to the difficulties experienced in the production of nuclear markers. The absence of mutant drug resistant phenotypes in the selection experiments may have been due to limitations in the techniques being used but if the organism were diploid only dominant mutations, which are generally rare compared

with similar recessive mutations, could be expected to yield mutant phenotypes. On the evidence available it is impossible to define the nature of the stable changes to streptomycin resistance and dependence. The absence of segregation in the asexual progenies may be taken to indicate either that the organism is haploid and streptomycin resistance and dependence are determined by single chromosomal genes, or that streptomycin resistance and dependence are not nuclear markers but are stable, heritable cytoplasmic states or cytoplasmic mutations induced or selected by streptomycin.

The frequent temporary adaptation to a variety of drugs and the abundance of spontaneous and induced morphological changes which do not appear to behave as would be expected if determined by chromosomal genes strongly suggests that the cytoplasm can exist in a number of states, at least some of which are heritable. Changes from one state to another seems to occur spontaneously but may be selected or induced by various environmental agents. It is not unlikely that cytoplasmic differences as well as genotypic differences in a plant pathogenic fungus are subjected to selection pressures during growth on various hosts. The race changes of P. infestans enabling avirulent races to become virulent and attack resistant potato varieties have usually been attributed to gene mutations but it also seems possible that changes in the cytoplasm selected or even induced by a resistant plant could be responsible for changes in virulence of the pathogen.

Proof of the existence of a system of cytoplasmic hereditary determinants can only be obtained when known nuclear markers can be used in appropriate tests.

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